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A Vibrio Associated with Swine Dysentery

L. P. DOYLE, B.S.A., M.S., Ph.D., D.V.M.

Lafayette, Indiana

SWINE DYSENTERY or bloody diarrhea has been recognized by a few investigators and by farmers for a number of years. The disease was first described more than twenty years ago by workers at the Indiana Agricultural Experiment Station.[1]. The slowness with which swine dysentery has been recognized is probably largely due to the fact that the specific cause has not hitherto been determined. Progress toward the proper recognition of dysentery as a disease entity, doubtless, also has been retarded by its inclusion in the catchall group of intestinal affections known as necrotic enteritis.

Dysentery can be readily reproduced by feeding a portion of the colon or the bowel discharge from affected hogs. In a number of trials, the colon was the only organ found capable of causing the disease when fed to susceptible animals. In one case, the colic lymph glands were carefully dissected out and fed to a susceptible pig without causing the disease, while the colon wall from the same animal caused dysentery when fed to another pig. These findings, together with the fact that the characteristic pathologic changes of dysentery are limited largely, if not entirely, to the colon, suggest that the disease is a localized infection. Hence, it appears that attempts to isolate the causative agent should be directed to an examination of the colon flora.

On microscopic examination of smears of mucus from the bowel of a dysentery hog, large numbers of vibrios are found. Sometimes, enormous numbers of these microörganisms are found in a "fish in the stream" arrangement. Moreover, microscopic examination of properly stained sections of the colon shows numerous vibrios deep in the crypts and at times invading the tissue, particularly the superficial portions of the mucosa. Apparently, at least two morphologic types of vibrios are recognizable in the colon of a dysentery hog. One is a rather large coarse form, while the other is a finer form. It has been known for a number of years that at least one form of vibrio may occur as a normal inhabitant of the hog's intestine.

A vibrio, in apparently pure culture, has been isolated from the colon mucosa of dysentery hogs. The most satisfactory method tried was as follows: The colon was opened so as to expose the mucosa. A portion of the surface of the mucosa was scraped with a scalpel in order to remove exudate and some of the superficial mucosa. With fine scissors a small piece of mucosa was snipped out of the scraped area. By means of an inoculating needle, this small piece of mucosa was streaked on the surfaces of several blood-agar plates. The blood-agar plates were inverted and placed in a widemouth jar. About 10 to 15 per cent of carbon dioxide was added, the jar was sealed, and then incubated at 37.5 C.

It required about forty-eight hours for

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Department of Veterinary Science, Purdue University Agricultural Experiment Station.

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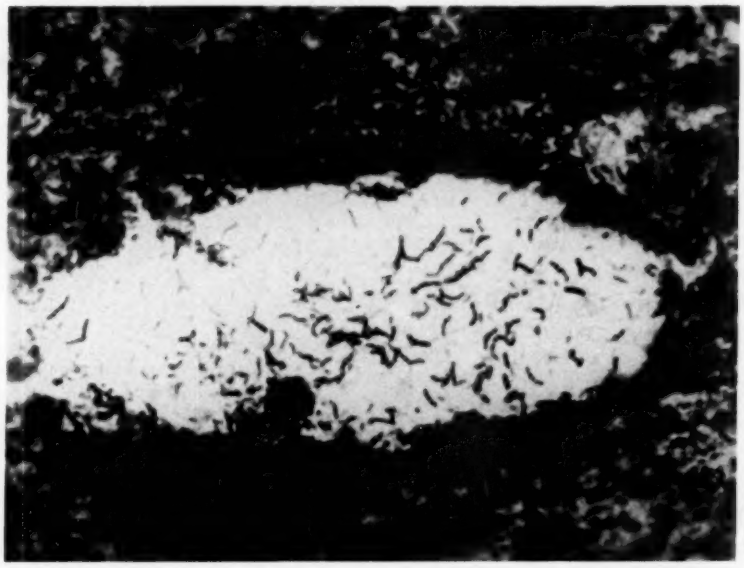
the vibrio to produce easily recognizable growth. Inoculated plates incubated in ordinary atmosphere failed to produce appreciable growth of vibrios. Small young colonies appeared translucent, but became more opaque and somewhat grayish as the amount of growth increased. The outlines of the larger colonies were somewhat irregular. Frequently, there was a tendency for the long axis of the colony to follow the needle stroke on the surface of the medium. The length of these elongated colonies was sometimes as much as 5 mm.

or Petri dishes were used in this manner, the vibrios made little or no growth in forty-eight hours. When plate cultures were stored in a refrigerator the vibrios died out by the end of several days.

The feeding of cultures of vibrios to pigs usually has been followed by diarrhea. Of 8 pigs fed cultures, 6 developed diarrhea. Usually, the diarrhea appeared at the end of three to five days. One of the animals did not show noticeable symptoms until the twenty-fourth day after being fed, when it developed well-marked dysentery.



A photomicrograph of a section of colon of a dysentery pig, showing numerous vibrios in the crypt. Vibrios are also present in the tissue some distance from the crypt. A silver nitrate impregnation method was used in the preparation of the tissue.

After Doyle

Transfer of the growth to new blood-agar plates was followed by fairly abundant growth when incubation was carried out in an atmosphere containing carbon dioxide. The addition of fresh defibrinated blood appeared to facilitate growth. Usually, no special difficulty was encountered in obtaining apparently pure cultures of vibrios when growth occurred. Sometimes, however, the microörganisms failed to grow on the streaked plates, suggesting that the growth requirements had not been fully met. Primary isolation was also obtained by sealing shallow inoculated blood-agar plates with parafilm and then incubating for forty-eight hours. When deep plates

In the others, the diarrhea varied from slight to well-marked, and usually lasted from three to five days. One pig had two recurrences of diarrhea at intervals of about one week.

With one exception, blood-agar plate cultures were used in the culture feeding trials. In one trial, cultures in the soft liver-infusion, agar medium described by Plastridge and Williams[2] were used. The growth from five or six 9-centimeter Petri dishes was suspended in nutrient broth, mixed with ground feed and then fed to a pig. When Plastridge's and Williams' medium was used, the growth in 19 tubes was fed to 1 pig. In this medium, the apparent

growth was limited to a shallow zone at the top of the column of medium; hence, the pig probably received not more than 15 to 20 cc. of culture.

The diarrhea which occurred following the feeding of vibrio cultures was not unlike that observed in some animals following the feeding of dysentery colon. As a rule, however, the diarrhea was less severe and there was usually less blood and mucus than are observed in typical cases of dysentery caused by feeding affected colon.

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The Effect of Chemotherapeutic Agents on the Bovine Mammary Gland

II. The Effect of Tyrothricin

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IN A PREVIOUS publication,[1] the effect of Novoxil on the normal bovine mammary gland was reported. The effect of tyrothricin was studied under similar conditions and is reported herein.

Little[2] found that gramicidin had some therapeutic value in the eradication of *Streptococcus agalactiae* from mastitis in cows, although repeated treatments were necessary to sterilize some quarters. These repeated treatments stimulated the production of fibrosis and resulted in a decrease in milk secretion. Little[3,5] determined that gramicidin suspended in mineral oil proved satisfactory for injection into udders. Little[4] stated that much remains to be learned concerning the action of gramicidin and, therefore, concerning the proper method of treatment. In selected cases, the dilute aqueous solution of gramicidin may offer advantages over the gramicidin-oil mixture. In general, the reaction of the udder to the injection of from 20 to 80 mg. of gramicidin-oil mixture was so mild that it was possible to repeat treatments on the second or third day. Schalm[6] found tyrothricin the most effective of the agents employed in the treatment of lactating udders. On the other hand, it was potentially the most irritating. The dosage must be adjusted carefully to the activity of the udder and the severity of the inflammatory process. The difference between gramicidin and tyrothricin, commonly used synonymously, is discussed in an editorial.[7] Favorable results with the use of gramicidin and tyrothricin were reported by Tripp,[8] Martin,[9] and Bryan.[10] Martin[11] later reported on unfavorable results with tyrothricin, and Bryan[10] found that the use of sterile mineral oil as a vehicle for administering the tyrothricin proved more irritating than the aqueous solutions.

The purpose of this study was to determine the effect of infusions of tyrothricin* upon the apparently normal mammary tissue.

MATERIALS AND METHODS

It was decided to adopt a plan similar to that in a previous report on Novoxil.[1]

There were two series of experiments and the pertinent data on the cows used are presented in table 1. In the first experiment, 2 cows with negative mastitis histories were used. The milk of all quarters was normal to all chemical and bacteriological tests. Cordy thickenings were palpable in all teat canals of cow 507. After a nine-day period, during which time the udder and milk were studied for preinfusion levels, the RF and RR quarters, only, were infused with 40 mg. each of tyrothricin as an oil mixture (40 cc.) immediately after milking, according to the technique of Little, *et al.*[3] Cow 507 was slaughtered forty-six days after infusion. In the second experiment, 2 cows were used and after a twelve-day period for the determination of preinfusion levels, the RF, LF, and LR quarters were infused with the tyrothricin for four successive days. Milking was continued on regular schedule, following infusions. Samples of milk were taken aseptically for bacteriological examination and other samples were obtained for the chemical and physical examinations.† Biopsies were made on these cows and, since an unfavorable reaction followed several biopsies and since the

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From the Division of Veterinary Medicine, University Farm, St. Paul, Minn.

*The supply of tyrothricin powder used in this experiment was made available through the courtesy of Merck & Co., Inc.

†These determinations were carried out by the Division of Dairy Husbandry.

milk was intermittently abnormal, they were retained for 126 days after infusion before slaughter.

The procedure and technique followed in the performance of the biopsies on the quarters are described in a previous publication.[12] In experiment 2, biopsies were performed on the RF (infused) and RR (not infused) quarters twenty-four hours after the last infusion (4

TABLE 1—Data on the Experimental Animals Used in the Two Experiments

Experiment	Cow No.*	Breed	Age yr.	Stage of Lactation mo.	Pregnant mo.	Quarters Infused
1	468	Holstein	3½	8.5	5	2
	507	Holstein	3	7.5	5	2
2	405	Holstein	6	4.0	open	3
	476	Holstein	4½	1.5	open	3

*These cows were found to be free from Bang's disease and tuberculosis.

successive daily infusions). A biopsy was performed on the LR quarter of cow 405 five days after the last infusion. The LF quarters remained as controls (table 5).

Observations

The effects of infusions upon the 4 cows are as follows:

1) *Physical Changes of the Udder and Teats.*—With the single infusion, the quarters were noticeably swollen three hours after infusion. This firmness involved the whole quarter; however, the subcutis softened up about nine hours after infusion so that the firmness was present only in the parenchyma. Three days after infusion, the gland was normal in appearance but when palpated, edema was noted. These changes were essentially the same as reported in the Novoxil experiment. Following the series of 4 infusions, tenderness was observed at the evening milking (ten hours after infusion) and the swelling decreased after the first infusion, despite the second, third, and fourth infusions. About three days after the last infusion, the swelling was greatly reduced and only a slight edema could be palpated in the parenchyma. With the 1 infusion (experiment 1), no definite anatomical changes could be palpated in the parenchyma before slaughter (forty-six days after infusion). The cord-like thickenings in the teat canals of cow 507 had not increased in size. With the 4 successive daily infusions, some fibrosis and edema were palpable in the gland cisterns of the LF and LR quarters (biopsy not performed), respectively, of cow 476, but neither was apparent before slaughter (table 5). The biopsies had a severe reaction on the RR quarters (not infused) of both cows, with abscessation resulting in in cow 476. Two biopsies made on the RF quarter of cow 476 were unsuccessful because of hemorrhage.

TABLE 2—The Effect of Tyrothricin upon the Physical Appearance of the Milk

Experiment	Cow	Quarter	Character of Secretion after Infusion*	Days after Infusion for Normal Appearing Milk
1	468	RF	stringy clots, yellow flakes, sediment	5
		RR	stringy clots, yellow flakes, sediment	2
		LF, LR	normal	not infused
	507	RF	stringy clots, yellow flakes, sediment	4
		RR	white flakes, serum	3
		LF, LR	normal	not infused

*About 10 cc. portion of foremilk refrigerated for eighteen hours.
In this experiment, the RF and RR quarters were infused with a 40 cc. dose. No biopsies were performed.

2) *Body Temperatures.*—In experiment 1, the hourly temperature readings revealed a rise of 2.2 and 1.4 F. in cows 468 and 507, respectively, nine hours after infusion. The return to the preinfusion level occurred one day after the infusions. In experiment 2, the first infusion resulted in a 1.0 F. rise five hours after infusion in both cows with a return approximately to the preinfusion level before the second infusion. This infusion brought a 2.0 and a 4.2 F. rise in cows 405 and 476, respectively, about five hours after the infusion. Again the temperatures were back nearly to the preinfusion levels before the third in-

fusion. This infusion elicited only a 1.0 F. rise in cow 405, five hours after the infusion. The last infusion brought no temperature response.

3) *Appetites.*—There was only a slight impairment of appetite in the cows in experiment 2 and this occurred during the temperature rise.

4) *Changes in the Milk.*—(*a*) Appearance and Volume: At the first milking (nine hours after infusion), the secretion was thick and contained stringy clots, yellow flakes, and sediment.

It was found (table 2) that with one dose (40 cc.), it took an average of three and six-tenths days, following infusion, for the milk to return to its normal appearance; with the 160 cc. dose (4 successive daily infusions), eight days. However, as can be seen in table 3, the milk was abnormal at various times in all quarters, including those not infused. Bloody milk was seen in 5 of the 6 quarters on biopsy.

The effect of tyrothricin upon the milk

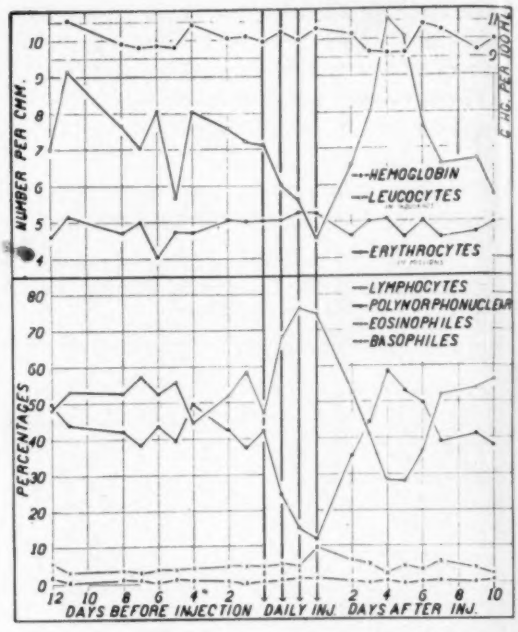

Fig. 1—Blood picture of cows 405 and 476 (experiment 2) before and after infusion with tyrothricin (4 successive daily infusions), averaged from one daily sample per cow.

TABLE 3—Effect of Tyrothricin and Biopsies upon the Physical Appearance of the Milk

EXPERIMENT	COW	QUARTER	CHARACTER OF SECRETION AFTER INFUSION*	DAYS AFTER LAST INFUSION FOR NORMAL APPEARING MILK	LATER INTERMITTENT OCCURRENCE OF ABNORMAL MILK AFTER INFUSIONS†	BLOODY MILK AFTER BIOPSIES DAYS
2	405	RF	cream clotty, milk thin with white or yellow sediment, oil on milk 2nd and 3rd day	9	19 days between 24th and 92nd day	1
		RR	brown sediment	1 not infused	7 days between 8th and 60th day	1
		LR	cream clotty, milk thin with sediment	9	21 days between 24th and 79th day	1
		LF	cream clotty, milk thin with sediment	9	7 days between 27th and 67th day	
	476	RF	cream clotty, milk thin with sediment	9	16 days between 27th and 67th day	8 unsuccessful
		RR	cream clotty, milk thin with yellow and white sediment	9 not infused	13 days between 26th and 67th day	1
		LR	cream clotty, milk thin with yellow and white sediment	6	14 days between 20th and 62nd day	
		LF	cream clotty, milk thin with sediment	6	12 days between 26th and 74th day	

*First 10 cc. sample of foremilk refrigerated for eighteen hours. †White flakes usually; yellow flakes, sediment, occasionally.

In this experiment, the RF, LF, and LR quarters received 4 successive daily infusions (160 cc.). Biopsies were made on the RF and RR quarters on both cows twenty-four hours after last infusion. A biopsy specimen was taken from the LR quarter of cow 405 five days after the last infusion.

production is shown in table 4. In neither experiment had the milk production returned to the preinfusion level at the end of twenty days, nor 126 days after infusion (experiment 2). In experiment 1, the cows were in late lactation and, therefore, the preinfusion level could not be expected.

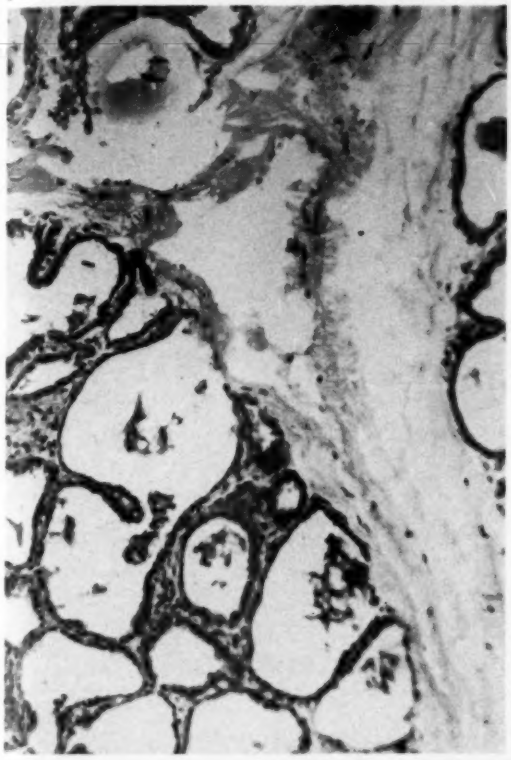

Fig. 2—Mammary gland (cow 405, RF quarter). Biopsy section taken four days after first infusion (one day after last infusion). This shows the presence of polymorphonuclear leucocytes, desquamated epithelium and concretions in some of the alveoli. There is also a lymphocytic infiltration, fibroblastic proliferation and congestion in the interalveolar spaces. x150. Hematoxylin and eosin stain.

b) Chemical Analyses: In an analysis of the data on the composition of the milk, significant differences were noted, mainly in the per cent of chlorides and lactose, as was observed in the Novoxil experiment.

c) Leucocytes: The leucocyte counts of the milk indicated a severe tissue reaction following infusion, as was noted in the Novoxil experiment. The second day after the single infusion, the leucocytes were approximately 10,000,000 and 7,000,000 per cc. in cows 468 and 507, respectively, in the quarters infused. In the ten days following infusion, the count rapidly decreased to about 1,000,000 per cc. The preinfusion status had been attained at the end of the experiment (forty-six days) in 3 of the 4 quarters on test. In experiment 2, one day after the first infusion the leucocyte count averaged about 7,500,000 per cc. for the 6 quarters on test. Although the biopsies produced a severe reaction in the RR quarters, no appreciable leucocyte increase was noted. Following infusion until slaughter (126 days), the leucocyte counts were intermittently high in the RF and LR quarters of cow 405 and they were accompanied by abnormal milk.

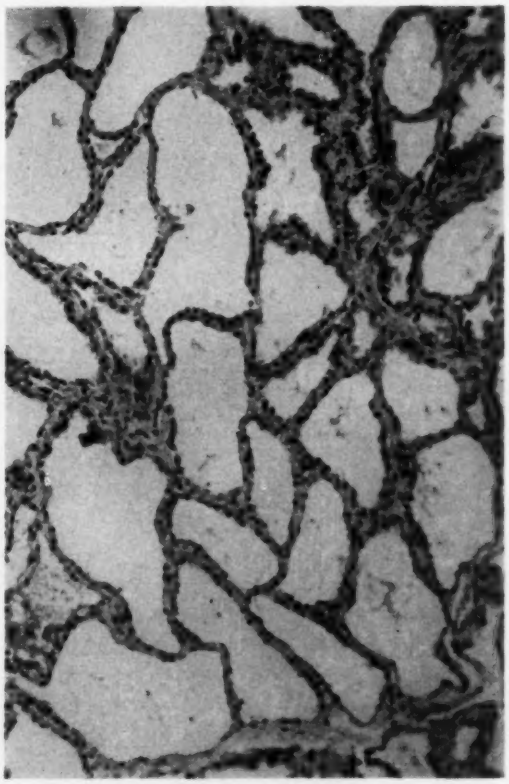

Fig. 3—Mammary gland (cow 405, RR quarter, not infused). Biopsy section taken at same time as that shown in figure 3. The alveoli appear normal. A few foci of fibroblastic activity occur in the interalveolar spaces. x150. Hematoxylin and eosin stain.

d) Bacteria: Bacteriological examinations of the milk of all quarters on test revealed it relatively free from any demonstrable bacteria during the course of the experiments.

TABLE 4—Effect of Tyrothricin upon the Milk Production of Infused Quarters

Experiment	Cow	No. of Quarters Infused	Months in Lactation	Milk Production in lbs. per Day						
				Day Before Infusions	Days after Infusions					
					1	2	5	10	20	126
1	468	2	8.5	2.6	1.3	1.8	1.6	1.7	1.7	—
	507	2	7.5	5.7	3.1	3.0	4.4	4.4	3.1	—
2	405	3	4.0	13.0	8.2	9.2	7.7	9.1	10.6	7.6
	476	3	1.5	9.5	6.3	8.0	8.3	6.8	7.6	5.2

In Experiment 5, the lb. of milk are computed for days after the last infusion in the series.
In Experiment 4, the RF and RR quarters were infused with one 40 cc. dose.
In Experiment 5, the RF, LF, and LR quarters received four successive daily infusions (160 cc.).

5) *Blood.*—Figure 1 shows the preinfusion and postinfusion levels of the differential blood cell counts. Following the infusion, a total leucopenia was noted in which the relative lymphocytosis was slight and the eosinophilia marked. Seven days after the last infusion, the preinfusion levels had been attained. As in the Novoxil experiment, the biopsies did not superimpose any significant changes on the postinfusion hemogram.

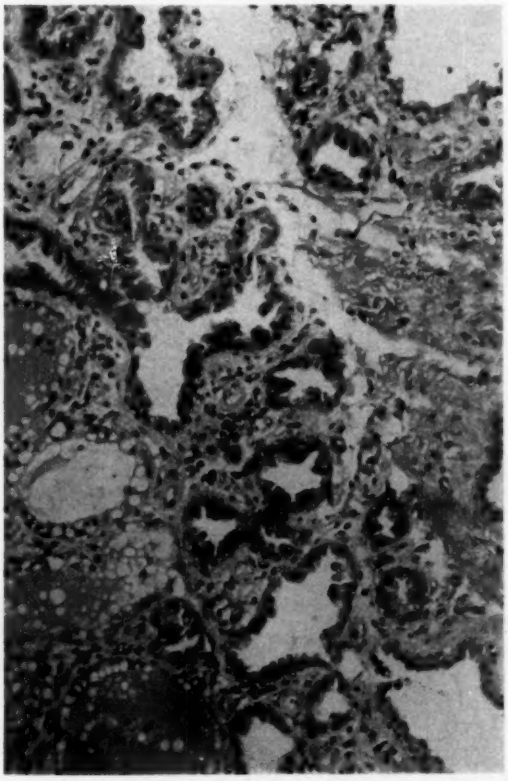

Fig. 4—Mammary gland (cow 405, LR quarter). Biopsy section taken eight days after first infusion (five days after last infusion). There are numerous polymorphonuclear leucocytes present in the alveoli and ducts and a desquamation of epithelium. Lymphocytic infiltration and fibroblastic proliferation in the interalveolar spaces are also noted. x150. Hematoxylin and eosin stain.

6) *Urine.*—No urinary tract changes were observed following the infusion of tyrothricin.

7) *Biopsies on the Udder.*—The tissues were fixed in 10 per cent formalin and stained with hematoxylin and eosin. Biopsy sections of the RF quarter (cow 405), four days after the first infusion, showed numerous polymorphonuclear leucocytes in a few alveoli. There was also some interstitial edema, capillary congestion, interalveolar lymphocytic infiltration, and proliferation of fibroblasts. Numerous concretions were also present (fig. 2). A biopsy on the RR

TABLE 5—The Effect of Infusions and Biopsies on the Quarters as Determined by Palpation Fifty-two Days after the Last Infusion

Experiment	Cow	Quarter	Total Amount Infused (cc.)†	Biopsy	Clinical Changes*
2	405	RF	160	yes	none
		LF	160	no	none
		RR	control	yes	none
		LR	160	yes	numerous small nodules in gland cistern
	476	RF	160	yes	none—2 unsuccessful biopsies
		LF	160	no	some fibrosis in gland cistern
		RR	control	yes	large encapsulated abscess
		LR	160	no	some edema in gland cistern

*The scar tissue at the biopsy site was still evident in all cases. †Each cc. containing 1 mg. of tyrothricin.

quarters (not infused), taken at the same time, showed apparently normal alveoli (fig. 3). A biopsy of the LR quarter, eight days after the first infusion, showed polymorphonuclear leucocytes present in alveoli and ducts, some lymphocytic infiltration and

Fig. 5—Mammary gland (cow 507, RF quarter, no biopsy). Section taken after slaughter (forty-six days after infusion) showing apparently normal alveoli. x60. Hematoxylin and eosin stain.

fibroblastic proliferation, and also some desquamation of the alveolar epithelium (fig. 4).

8) *Udder After Slaughter.*—Cow 507 (1 infusion) was slaughtered forty-six days after infusion. A section of the RF parenchyma showed apparently normal alveoli (fig. 5). There was a verrucose lesion present in the RR teat canal which could be palpated before infusion. Microscopically, this lesion showed epithelial hyperplasia and numerous concretions. This type of pathology has been described elsewhere.[13]

As no tracer element such as the silver in the Novoxil experiment was present in the tyrothricin, sections of tissue for the demonstration of pathological changes could be taken only at random. Palpation did not reveal any extensive changes and as several biopsies brought on severe reactions (RR quarters not infused) the cows were retained for 126 days after infusion before slaughter (table 5). The RF quarter of cow 405, on which a biopsy was made,

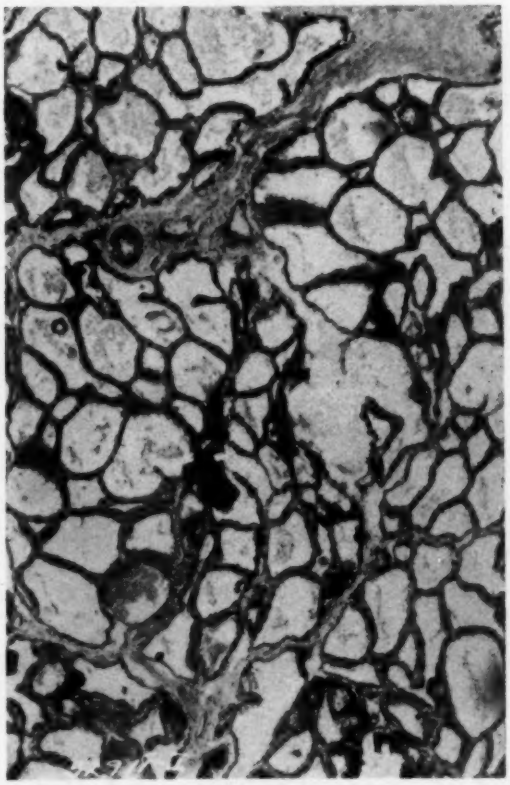

Fig. 6—Mammary gland (cow 405, RF quarter, biopsy). Section taken after slaughter (126 days after last infusion) showing apparently normal alveoli. There is some fibroblastic proliferation present in the interalveolar spaces. Numerous concretions can be seen. x60. Hematoxylin and eosin stain.

showed apparently normal alveoli (fig. 6), after slaughter. The LF quarter of cow 476, on which a biopsy was not made, also showed apparently normal alveoli (fig. 7). The LR quarter of this cow, on which biopsies also were not made, showed a low grade inflammation (fig. 8).

SUMMARY AND CONCLUSIONS

In a series of two experiments, a total of 10 apparently normal quarters of 4 cows were infused with tyrothricin-oil mixture to determine its effect upon normal lactating mammary glands. Four quarters of 2 cows were infused with 40 cc. doses and 6 quarters of 2 other cows were infused with four 40 cc. doses on successive days, totaling 160 cc. Each cc. contained 1 mg. of tyrothricin.

In both instances, the infusions produced a condition simulating acute mastitis as was observed with Novoxil. The glands became enlarged, tender, and firm; the milk became abnormal in consistency; and the percentages of the milk chlorides increased and those of the lactose decreased. This was accompanied by an increase in polymorphonuclear leucocytes in the milk.

After 4 successive daily infusions, the milk production had not returned to the preinfusion status 126 days after the last infusion (end of experiment). Abnormal milk was observed intermittently as long as the ninety-second day after infusion. This occurred in all infused quarters and also in the two quarters which were not infused.

As no tracer element such as the silver in the Novoxil experiment was present in the tyrothricin, its localization in the gland tissue could not be determined.

No apparent permanent changes were noted macroscopically or microscopically in the udder forty-six days after infusion (1 infusion) and 126 days after the fourth infusion (4 successive daily infusions) of tyrothricin.

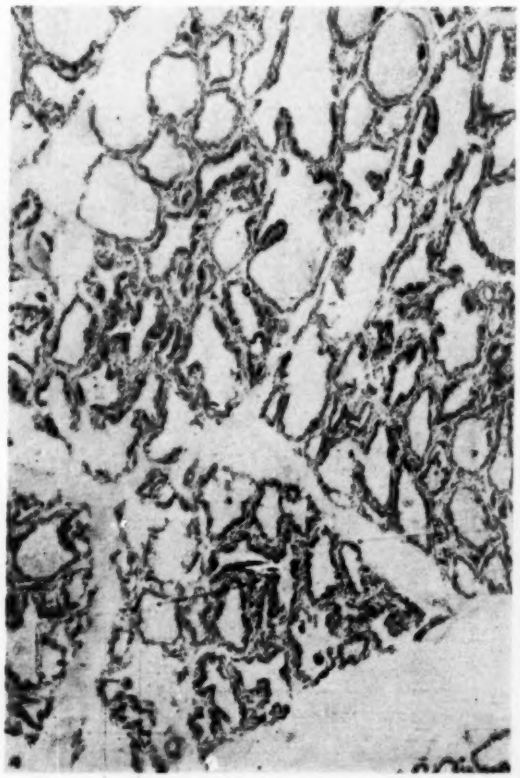

Fig. 7—Mammary gland (cow 476, LF quarter, no biopsy). Section taken after slaughter showing apparently normal alveoli. x60. Hematoxylin and eosin stain.

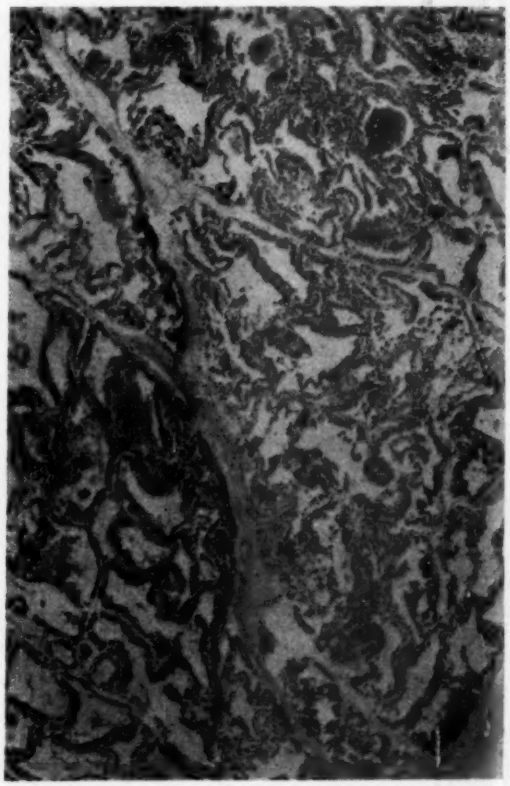

Fig 8—Mammary gland (cow 476, LR quarter, no biopsy). Section taken after slaughter. A low grade inflammation is present in the interalveolar tissue. x60. Hematoxylin and eosin stain.

ACKNOWLEDGMENTS

In this series of experiments, the writers are grateful to the Division of Dairy Husbandry for the chemical determinations on the milk, V. P. Hollis and his staff for the photography, Florence Jones for her work on the blood determinations, and Jean McIntyre Feist for her assistance in the presentation of the data.

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Attempts to Demonstrate Passive Immunity in Bovine Trichomoniasis

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RIEDMULLER,[1] Witte,[2] Endress,[3,4] Kerr and Robertson,[5] and others have demonstrated that infection of cattle with *Trichomonas foetus* results, in some cases, in the production of serologically detectable antibodies. This work, however, has not led to a diagnostic test, satisfactory for general use. Resistance to reinfection has been shown to vary in degree and in duration by Andrews,[6] Andrews and Miller,[7] Rees and Garlick,[8] and others. Since none of them dealt with the passive transfer of immunity, it becomes of interest to determine whether it is possible to immunize female cattle, passively, against infection with *T. foetus*.

MATERIALS AND METHODS

Four previously uninfected heifers, 189, 186, 206, and 178, were given intravenous injections of serum or defibrinated blood obtained from the resistant cows described below and two heifers, 187 and 190, were used as controls. All except one, 178, were from 4 to 7 months of age at the time these experiments were undertaken. Three to 5 doses were given to each heifer during a ten-day period, preceding exposure to infection, with the exception of heifer 206, which received 8 injections during the month preceding exposure to infection. Heifer 189 received injections of defibrinated blood instead of serum. Following the exposure, three of the heifers, 186, 206, and 178 received 11, 5, and 17 doses of serum during periods of forty-five, seventeen, and forty-eight days, respectively.

Three cows, each of which had experienced one or more infections with *T. foetus*, were used as sources of blood. Prior to the time they were used for this purpose, all 3 animals had remained negative for *T. foetus*, in spite of repeated exposures by intravaginal inoculations of trichomonad cultures, and by services of infected bulls. The blood was allowed to stand overnight and the serum was injected the following day. The amount of serum injected varied from 100 to 200 cc., the average dose being approximately 160 cc. One cow was used as the source of defibrinated blood and serum for 2 heifers and each of the other 2 cows was used as a source of serum for only 1 heifer.

All heifers, including the controls, were exposed to *T. foetus*, 5 by intravaginal inoculation of 35 to 85 cc. of culture fluid containing several millions of active trichomonads, and 1 (178) through coitus with an infected bull. The strain of trichomonads used had been maintained in culture about two years and was bacteria-free during most of the time covered by this experiment. Preceding and following exposures, vaginal samples were collected daily from each heifer and examinations for trichomonads were made by direct and culture methods.

All animals were reëxposed to infection. Heifers 186 and 206 that had not become infected as the result of the first exposure were reëxposed eight months and nineteen days, respectively, following the first exposure. The other 4 (187, 189, 190, and 178) were reexposed after periods ranging from nineteen days to three months, following the last positive examination.

Three methods of reëxposure were employed: heifer 206 by inoculation with preputial flushings from an infected bull; heifers 186 and 178 by coitus with infected bulls; heifers 187, 190, and 189 by intravaginal inoculation of trichomonads in culture fluid, as in the first exposure. Heifer 206, that had not become infected following her first exposure nineteen days previously, received serum at regular intervals up to the time of the second exposure and was given 5 doses of serum during the fifteen days following the second exposure. The other heifers received no serum, either immediately before or after the second exposure.

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The experimental work reported in this paper was planned and carried out by the senior author (Garlick) prior to his resignation in May, 1941. The results were interpreted and prepared for publication by the other authors.

RESULTS

The results shown for individual heifers in table 1 may be summarized as follows:

1) The 2 untreated controls (187 and 190) became infected as a result of intravaginal inoculation of trichomonads in culture fluid.

2) Two heifers (186 and 206) of the 4 which received injections of either serum or defibrinated blood did not become infected when exposed by the method which produced infections in the controls. These 2 heifers received injections of serum before and after inoculation.

3) Two of the treated heifers became infected. One of these (189) had received injections of defibrinated blood before the intravaginal inoculation of trichomonads; in this case, the prepatent period was significantly longer than those of the 2 controls, while the patent period was intermediate in length between those of the 2 controls. The other heifer (178), which was served by an infected bull, received injections of serum before and after exposure; in this case, the resulting infection, the prepatent period of which was normal for natural infections, developed into pyometra.

4) Upon being exposed a second time by intravaginal inoculation, 1 untreated control (187) became infected and the other (190) did not.

5) The 2 treated heifers (186 and 206), which had failed to become infected as a result of the first exposure, became infected as a result of the second exposure. One heifer (186) was served by an infected bull; no additional serum was given. The other heifer (206) was inoculated intravaginally with preputial flushings from an infected bull. The injections following the first exposure had been continued up to the time of the second exposure; 5 injections of serum were given thereafter. The patent periods of these infections were shorter than is usual for first infections.

6) The two treated heifers (189 and 178), which had become infected as a result of the first exposure, did not become infected following the second exposure. One heifer (189) was inoculated intravaginally with culture fluid; the other heifer (178) was served by an infected bull.

TABLE 1—Results of the Exposure of Treated and Control Heifers

Number and birth date Heifers	Number of injections given			Dates of 1st and last injection	1st Exposure			2nd Exposure		
	Defibrinated blood before 1st exp.	Serum			Date and method	Prepatent period (days)	Patent period (days)	Date and method	Prepatent period (days)	Patent period (days)
		Before 1st exp.	After 1st exp.							
187 (control) 11/5/38	—	—	—	—	6/24/39 Cultures intravag.	0	67	10/2/39 Cultures intravag.	7	28
190 (control) 1/3/39	—	—	—	—	5/15/39 Cultures intravag.	2	16	6/21/39 Cultures intravag.	No infection developed	
186 11/3/38	—	5	11	6/15/39 8/8/39	6/24/39 Cultures intravag.	No infection developed		2/17/40 Coitus Inf. ♂ 150	10	32
206 5/11/40	—	8	5	10/18/40 12/1/40	11/16/40 Cultures intravag.	No infection developed		12/5/40[1] Preputial flushings[2] intravag.	3	22
189 12/?2/38	5	—	—	5/23/39 6/2/39	6/2/39 Cultures intravag.	16	24	8/26/39 Cultures intravag.	No infection developed	
178 11/7/37	—	3	17	10/11/39 12/9/39	10/22/39 Coitus Infected ♂ 150	10	206[3]	8/30/40 Coitus uninfected ♂ 209	No infection developed	

[1]Note that injections of serum following first exposure were continued up to the time of the second exposure. Additional doses of serum, following the second exposure, were given on 12/7/40, 12/10/40, 12/13/40, 12/15,40, and 12/20/40.
[2]Preputial flushings from infected ♂ 208 intravaginally.
[3]Pyometra.

SUMMARY AND CONCLUSIONS

Attempts were made to transfer immunity passively from 3 immune cows to 4 susceptible heifers by the intravenous injection of defibrinated blood and serum. Two control heifers were not treated. All heifers were tested for susceptibility by exposure to trichomonads, by inoculations of cultures or preputial flushings, or through coitus.

Although the number of animals employed is too small to permit definite conclusions, the following generalizations appear to be justified.

1) The injections of serum from cows that had acquired immunity to trichomoniasis appeared to confer some resistance to infection with *Trichomonas foetus*, as shown by the fact that 2 of the 3 heifers given the injections of defibrinated blood or serum, and subsequently inoculated with culture (which produced infections in the 2 controls), did not become infected.

2) This treatment was not effective in the 2 cases in which exposure involved infection with trichomonads from a bull, as is shown by the fact that neither the heifer bred to an infected bull during treatment nor the heifer inoculated with preputial flushings immediately following treatment, was protected.

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Attempts to Develop Active Immunity to Bovine Trichomoniasis in Breeding Females by Inoculation Prior to Breeding Age

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THE WORK OF a number of investigators[1-9] has established that (a) infections with *Trichomonas foetus* in heifers and cows are usually self-limiting and ordinarily not responsible for any permanent impairment of the reproductive system; (b) infections are followed by variable periods and degrees of resistance to reinfection; and (c) following infection most heifers and cows conceive and complete successful pregnancies following the services of infected bulls without evidence of reinfection.

Since immunization prior to breeding age might have practical application, experiments were undertaken to determine whether it is feasible to immunize breeding females actively by intravaginal inoculations of *T. foetus* begun during calfhood.

MATERIALS AND METHODS

Female cattle 187, 189, 190, and 206 that had been used in previously reported work[10] were used in the experiments reported herein. These females had experienced their first infection when 4 to 7 months old as the result of inoculation with *T. foetus*. Female 189 had received injections of defibrinated blood from an immune cow prior to and following her first exposure to infection and female 206 had received serum from an immune cow prior to and following her first and second exposures. In only one instance did these injections appear to influence the development of infection.[10] The time elapsing between the preliminary injections and the subsequent exposures discussed in the present paper was such that the effect of any passively transferred immunity would have been lost. The remaining 2 females, 187 and 190, received no injections of either blood or serum.

The cultures used for intravaginal inoculation of the calves had been maintained for about two years and were bacteria-free during most of the period covered by this experiment. For each inoculation, 35 to 85 cc. of culture fluid containing several millions of active trichomonads were employed. The preputial flushings used varied greatly in quantity and concentration of trichomonads. The culture used for the intracervical test inoculation of female 190 was a recently isolated, bacteria-free strain.

Following the initial infection, subsequent inoculations were made up to the time of breeding to an infected bull. Females 187, 189, and 190, inoculated with culture, experienced 2 infections each; female 206, inoculated with preputial flushings, experienced 1 infection. In every case, the animal had experienced at least 1 inoculation which failed to produce infection prior to her service by an infected bull. Three infected bulls 193, 207, and 208 were used for the test exposures.

Vaginal samples were collected daily, except Sundays, in the periods immediately preceding and following each exposure and during and immediately following each infection. At other times, samples were collected weekly. Examinations for *T. foetus* were made by direct and cultural methods. Inoculations were given before and after puberty; the latter exposures were made during estrum.

RESULTS

The results of the test exposures are summarized in table 1. Detailed data for individual females are presented in the following protocols:

Protocol for Female 187.—When 7 months old, this animal was inoculated intravaginally with 56 cc. of culture containing approximately 5,000,000 *T. foetus* per cc. An infection developed; the vaginal specimens were positive irregularly over a period of sixty-seven days. Five weeks following the last positive finding this animal was inocu-

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The work was initiated by George G. Garlick prior to his resignation in May, 1941.

lated intravaginally with 35 cc. of culture containing approximately 20,000,000 *T. foetus* per cc. An infection developed; the vaginal specimens were positive irregularly over a period of thirty-five days. Eleven months following the last positive finding this animal was inoculated intravaginally with 80 cc. of culture which contained an undetermined number of *T. foetus* per cc.; no infection developed. At the time this animal was to be bred for her first calf, an infected bull was not available; therefore, her resistance to infection could not be tested at that time. She was served once by an uninfected bull and she conceived and completed a normal pregnancy. When approximately 3 years old, this animal was served by infected bull 207 and developed a trichomonadal pyometra which terminated spontaneously in the fourth month. She was irregularly positive for trichomonads on vaginal examination, over a period of 107 days. Twenty-three days after the last positive examination this animal was served by infected full 193. Thirty days later she was again served by bull 193 from which service she conceived and completed a normal pregnancy. Examinations following these latter exposures revealed no infection.

Protocol for Female 189.—When 6 months old, this animal was inoculated intravaginally with culture of *T. foetus* (number of organisms not determined). An infection developed; the vaginal specimens were irregularly positive over a period of forty days. Prior to and following her first exposure this animal received intravenous injections of defibrinated blood from a trichomonad-resistant cow. This treatment had no obvious effect upon the course of

TABLE I—Initial and Subsequent Test Exposures of 4 Females to Trichomonas Foetus

	Initial Exposures			Test Exposures			
	Date of Inoculations		Result; Duration of Infection in Days	Date of Exposures		Result; Duration of Infection in Days	Result of Service
	Culture	Preputial Flushings		Service of Bull (No.)	Inoculation		
Female No. 187 born 11/5/38	6/24/39	—	Inf.; 67	11/8/40 Uninf. 209	—	—	Normal pregnancy
	10/2/39	—	Inf.; 35				
	9/27/40	—	Neg.; —	12/30/41 Inf.207	—	Inf.; 107	Pyometra[1]
				5/9/42 Inf.193	—	Neg.; —	No evidence of conception
				6/8/42 Inf. 193	—	Neg.; —	Normal pregnancy
Female No. 189 born 12/22/38	6/2/39[2]	—	Inf.; 40	9/22/41 Inf. 208	—	Inf.; 52	No evidence of conception
	8/26/39	—	Neg.; —				
	10/2/39	—	Inf.; 65	12/8/41 Inf. 208	—	Neg.; —	No evidence of conception
	9/24/40	—	Neg.; —	1/31/42 Inf. 207	—	Neg.; —	Normal pregnancy
	12/26/40	—	Neg.; —				
Female No. 190 born 1/3/39	5/15/39	—	Inf.; 16	1/19/42 Inf. 207	—	Neg.; —	No evidence of conception
	6/21/39	—	Neg.; —				
	7/21/39	—	Neg.; —	3/5/42 Inf. 207	—	Neg.; —	No evidence of conception
	8/26/39	—	Neg.; —	4/7/42 Inf. 207	—	Neg.; —	No evidence of conception
	10/2/39	—	Inf.; 25				
	1/3/41	—	Neg.; —	4/27/42 Uninf. 210	—	—	—
	12/30/41	—	Neg.; —				
				—	5/9/42[4]	Inf.; 16+[5]	—
Female No. 206 born 5/11/40	11/16/40[3]	—	Neg.; —	2/10/42 Inf. 207	—	Neg.; —	No evidence of conception
		12/5/40[3]	Inf.; 25				
		11/8/41	Neg.; —	2/27/42 Inf. 207	—	Inf.; 15	Normal pregnancy
		11/26/41	Neg.; —				
		1/21/42	Neg.; —	2/1/43 Inf. 207	—	Inf.; 292+	Pyometra[6]

[1]Terminated spontaneously in 4th month. [2]Received intravenous injections of defibrinated blood from an immune cow. [3]Received intravenous injections of serum from an immune cow. [4]10 cc. bacteria free culture intracervically. [5]Slaughtered on the 16th day. [6]Terminated by treatment.

the infection. Six weeks following the last positive examination this animal was inoculated with 40 cc. of culture containing 5,000,000 *T. foetus* per cc.; no infection developed. Five weeks following the last exposure she was inoculated intravaginally with 35 cc. of culture containing 20,000,000 *T. foetus* per cc. An infection developed; the vaginal specimens were irregularly positive over a period of sixty-five days. Ten months after the last positive examination this heifer was inoculated intravaginally with 85 cc. of culture containing an undetermined number of *T. foetus;* no infection developed. Three months after the last exposure she was again inoculated intravaginally with 50 cc. of a culture containing an undetermined number of *T. foetus*; no infection developed. Ten months after the last inoculation with culture material this heifer was served by infected bull 208 and developed an infection which persisted for fifty-two days. Three weeks after the last positive finding she was again served by the same infected bull. There was no evidence of either conception or infection. Eight weeks later she conceived and subsequently completed a normal pregnancy without evidence of infection, as a result of the service of infected bull 207.

Protocol for Female 190.—When 4 months old, this animal was inoculated intravaginally with an undetermined quantity of culture of *T. foetus.* An infection developed; the vaginal specimens were irregularly positive over a period of sixteen days. Three weeks following the last positive finding this animal was inoculated intravaginally with an undetermined quantity of a culture of *T. foetus*; no infection developed. Four weeks later she was inoculated intravaginally with 100 cc. of a culture containing 4,000,000 *T. foetus* per cc.; no infection developed. Five weeks later she was inoculated with 40 cc. of culture containing 5,000,000 trichomonads per cc.; no infection developed. Five weeks later she was inoculated with 35 cc. of culture containing 20,000,000 *T. foetus* per cc. An infection developed; the vaginal specimens were positive over a period of twenty-five days. Thirteen months following the last positive finding she was inoculated intravaginally with 50 cc. of culture containing an undetermined number of *T. foetus*; no infection developed. Twelve months later she was inoculated with 30 cc. of culture containing 8,000,000 *T. foetus* per cc.; no infection developed. Three weeks following this last inoculation she was served by infected bull 207 but failed to become either infected or pregnant. Similar results occurred six weeks later when she was served by the same infected bull. Three weeks later she was again served by infected bull 207 and did not become pregnant. Three weeks later she was served by an uninfected bull. Ten days after this last service she was in estrum as the result of enucleation of a corpus luteum. She was then inoculated intracervically with 10 cc. of a recently isolated bacteria-free culture of *T. foetus* containing 1,000,000 organisms per cc. This exposure resulted in an infection. She was slaughtered on the sixteenth day following exposure. Postmortem examination revealed trichomonads in great numbers on the surface of the endometrium. Histological study of the uterus revealed changes characteristic of a mild endometritis.

Protocol for Female 206.—When 6 months old, this animal was inoculated intravaginally with 50 cc. of a culture containing an undetermined number of *T. foetus.* Prior to and following the first and second exposures this animal received intravenous injections of serum from a trichomonad-resistant cow. Three weeks after the first exposure she was inoculated intravaginally with 30 cc. of saline flushings from the prepuce of bull 208 which contained 1 to 3 *T. foetus* per drop. No infection resulted from the first exposure, but following the second exposure an infection developed which persisted for twenty-five days. Ten months after the last positive examination this animal was inoculated intravaginally with 7 cc. of saline flushings from infected bull 208 which contained 10,000 *T. foetus* per cc.; no infection developed. Three weeks later she was again inoculated with the same number of organisms from the same bull; no infection developed. Two months later she was inoculated intravaginally with

1.5 cc. of saline flushings of the prepuce of infected bull 207; no infection developed. Three weeks later this animal was served by infected full 207. From this exposure an infection resulted, vaginal specimens being positive over a period of fifteen days. However, a normal pregnancy resulted from this service. Examination of the amniotic and allantoic fluids, and of the oral mucus and meconium, immediately following parturition, revealed no *T. foetus*. One year following her first breeding this animal was served by the same infected bull. Vaginal specimens were negative for seven weeks, at which time she became unavailable for further observations. At the time of anticipated parturition, examination of the uterus revealed a typical pyometra. Trichomonads were found later in the pus.

Discussion of Results

As shown in table 1, 2 females (187 and 189) that had experienced infection as calves, as a result of intravaginal inoculation with cultures of *T. foetus*, became infected following their first exposure through coitus with an infected bull. Each had proved refractory on one or more occasions in the interval between the early experimentally-produced infections and the natural exposures. This interval was relatively long in the case of female 187; a normal gestation occurred during this period. The 2 infections which developed following service by an infected bull were in course, duration, intensity, and effect upon fertility similar to infections which we have observed in previously uninfected females. It is apparent that inoculation with culture of *T. foetus* failed to produce effective resistance in these 2 animals.

In the case of female 190, which failed to become infected following exposure on three occasions through coitus with an infected bull, it appears that some resistance resulted from the earlier experimentally produced infections. However, this animal was not resistant to inoculation with large numbers of trichomonads, as shown by the fact that an infection developed following the intracervical inoculation of the recently isolated culture of *T. foetus*.

Female 206, which had been previously infected through inoculation with preputial flushings rather than culture of *T. foetus*, became infected when exposed through service of an infected bull. This was evidently a relatively benign infection, since it differed from the usual first naturally acquired infections in the following respects: It was of short duration; the trichomonad population did not reach a high level; and the pregnancy was not adversely affected. An infection followed an exposure one year later when she was served for her second calf. This infection was unique in this series in that, although vaginal samples were negative for seven weeks following exposure, a trichomonadal pyometra developed.

In general, these data indicate that infections in calves resulting from intravaginal inoculations of *T. foetus*, as used in these experiments, did not consistently produce resistance sufficient to protect against infection resulting from services by infected bulls approximately two years later. It is possible that the use of trichomonads that had been maintained in culture for a long time may have contributed to the failure to produce effective resistance to a natural infection.

It is possible, also, that an early, appreciable immunity, the presence of which was not checked in the present study, may have existed in the months immediately following the initial inoculations. Even if such a transient immunity were induced, it would be valueless from a practical standpoint if it failed to protect females when bred for their second and subsequent calves.

Summary and Conclusions

Attempts were made to immunize, actively, 4 breeding females by inoculating them 3 to 7 times intravaginally, prior to breeding age, with cultures or preputial flushing containing *Trichomonas foetus*. One to two years after the last infection produced by such inoculations, which was followed in the individual cases by 1 to 3 additional inoculations that gave negative responses, each female was exposed to infection

through coitus with an infected bull. Three of the 4 subjects became infected a total of 4 times as the result of exposures when served for their first and/or second calf. Of the 3 significant infections, 2 were characterized by pyometra. The fourth animal failed to become infected following 3 exposures through coitus, but subsequently developed an infection as a result of intracervical inoculation of trichomonads. It is concluded that although a temporary immunity is apparently induced by repeated inoculation, it is ineffective in protecting breeding females from naturally acquired infection as late as two years after the inoculation.

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Effects of the Intestinal Nematode, Trichostrongylus Colubriformis, on the Nutrition of Lambs

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IN 1933, STEWART[6] and Shearer and Stewart[4] published the results of nutrition experiments indicating that the digestibility coefficients of crude protein and crude fiber and the absorption of mineral matter were somewhat less in lambs harboring large numbers of gastrointestinal parasites than in the same lambs after they had lost their infections. This work led us to undertake experiments to ascertain the effect of heavy infections with single species of gastrointestinal parasites on the metabolism of sheep.

The first paper of this series[1] showed that *Cooperia curticei*, a relatively nonpathogenic intestinal nematode of sheep, had no effect on the digestibility coefficients or absorption of the various components of ingested feed. However, it apparently did affect the utilization of these components because the number of pounds of live weight gained per pound of feed ingested was reduced. This decrease in the efficiency of the infested animals in utilizing their feed was shown to be due to an increase in energy metabolism.

This paper deals with studies of the nutrition of lambs experimentally infected with the intestinal nematode, *Trichostrongylus colubriformis*. The clinical and pathological aspects of the disease produced by this nematode were published in an earlier paper.[2]

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MATERIALS AND METHODS

Six crossbred Hampshire-Southdown lambs (four wethers, No. 68, 70, 61, and 72, and two ewes, No. 57 and 58) were used in the present experiment. The lambs were born at the Beltsville Research Center, Beltsville, Md., on Feb. 26, March 16, 19, 31, and 31, and April 3, 1935, respectively. They were weaned when approximately 2 months old and were then given a feed mixture having a nutritive ratio* of 1:4.38. This mixture consisted of alfalfa meal, 27 lb.; ground oats, 10 lb.; corn meal, 5 lb.; bran, 3 lb.; and salt, ½ lb. In addition, all lambs were given an ample supply of water. Lambs 68, 70, 61, and 72 were placed on experiment on July 9, 1935; lambs 57 and 58, on July 8, 1935.

The metabolism cages were the same as those used in the work with *C. curticei*.[1] The technique employed in handling the lambs before and during the experiment was also the same as that in the previous experiment. However, the plan involving 3 sets of paired lambs which was originally carried out in the first experiment had to be abandoned because of the lack of males. This reduction in the number of animals was partly compensated for by using the lamb originally intended only for furnishing infective material, by infecting 1 of the control lambs late in the experiment, and by using 2 ewe lambs on which certain data could be obtained, but which could not be put into the metabolism cages because of the difficulty of collecting feces uncontaminated with urine from female sheep.

For the purpose of infecting the lambs, specimens of *T. colubriformis* were recovered from the small intestine of a sheep slaughtered at the Beltsville Research Center. The larvae obtained by culturing the eggs were administered by mouth in water to a parasite-free lamb. Later, the feces of this lamb containing eggs of *T. colubriformis* were cultured and the larvae used for infecting the experimental animals. The progress of the infections was followed by counting the number of worm eggs passed in the feces at two- to four-day inter-

*The ratio of the quantity of digestible protein to the quantity of digestible fat (x 2.25) and carbohydrate in a given food substance.

vals. The Lane Direct Centrifugal Flotation and Stoll Dilution Methods were used.

The chemical analyses differed from those of the previous experiment only in the substitution of the urea nitrogen determination for that of hippuric acid in the urine samples. The xanthydrol method was used for determining the quantity of urea present. The energy metabolism was calculated as in the preceding paper, the energy value of the feed being 1.048 therms per lb. (1 therm = 1,000 calories.)

Lambs 58 and 72 died of trichostrongylosis. The former died on October 4, and the latter on Nov. 16, 1935, the closing date of the experiment. Lambs 57, 61, 68, and 70 were slaughtered on this date, and slaughter and carcass grades were obtained on them. Postmortem examinations were made on all of the lambs, and the number and species of parasites found were recorded.

DATA ON EXPERIMENTS

Degree of Infection.—The number of infective larvae of *T. colubriformis* administered to the lambs, the duration of the diarrhea when this symptom was observed, and the number of trichostrongyles recovered *post mortem* are given in table 1.

TABLE 1—Degree of Parasitic Infection of Experimental Lambs

Lamb No.	Doses of Larvae	Period of Administration of Larvae	Total Larvae	Interval between Beginning of Infection and Onset of Diarrhea	Duration of Diarrhea	Interval between Beginning of Infection and Necropsy	Trichostrongyles Recovered
	No.		No.	Days	Days	Days	No.
57	102	7/8-11/11	112,750	..	..	127	7,004
58	88	7/8-10/3	440,000	35	53	88	117,756
68	123	3/13-11/11	138,250	..	..	126	8
70	119	7/9-11/11	63,000	..	..	126	4
72[a]	27	9/16-10/12	135,000	26	35	61	63,875
61	0	.....	.....	..	..	126[b]	0

[a]Lamb 72 was an uninfected control lamb from July 9 to Sept. 16.
[b]Interval from beginning of experiment to necropsy.

The data in table 1 show that relatively large numbers of infective larvae of *T. colubriformis* were administered to 5 of the lambs over a period of twenty-seven to 126 days and that as a result of the experimental infection diarrhea occurred in lambs 58 and 72. The lambs surviving the infection, and the control lamb, showed no clinical symptoms. Although relatively few trichostrongyles were recovered at the postmortem examination of the surviving lambs, the data in table 2 show that these parasites were present in considerable numbers during the experiment, as was indicated by the number of worm eggs passed in the feces.

A few specimens of nematodes belonging to other genera were also recovered. However, the number present was too small to be considered sufficiently important to influence the results of the work. They are not included in table 1.

Feed Consumption and Weights of Lambs.—The average daily feed consumption for each nutrition period and the growth curve of each lamb are shown in figures 1 and 2, respectively.

A comparison of these figures shows that the feed consumption of each lamb roughly parallels its growth curve. The data further show that lambs 58 and 72, which developed clinical symptoms of trichostrongylosis, reduced their daily feed consumption after infection, lost weight rapidly, and with the onset of diarrhea, weakened and died within a relatively short time, and that the weight curves of lambs 61, 70, and 68 are in inverse order to the magnitude of the infections received by them.

The differences in the appearance of the carcasses of lambs 61, the control, 70, and 68 are shown strikingly in figures 3 and 4. Lambs 61 and 70 received slaughter and carcass grades of "good +," whereas lamb 68 received a grade of "medium" in both classifications. Lamb 57, graded and slaughtered at the same time as the first 3 lambs, received "good +" in both classifications but was not photographed with them as it was not comparable in size.

Data on the number of pounds of feed consumed per 100 lb. of live weight gained are shown for both infected and control lambs in table 3.

These data show that the infected lambs consumed almost twice the quantity of feed as the controls for every 100 lb. of live weight gained. Although there was a wide

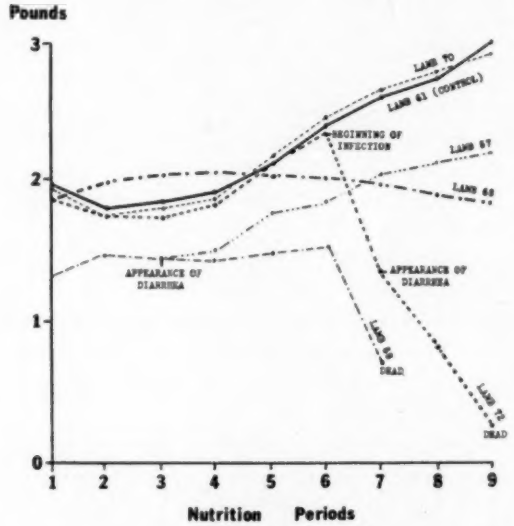

Fig. 1—Average daily feed consumption of infected and control lambs.

variation among the infected lambs in the number of pounds gained per pound of feed consumed, due to differences in the number of larvae administered, in the reaction of the individual lambs to the infection, and in the length of time the lambs harbored the infections, in no instance did a control lamb require as much feed as an infected lamb for the same amount of weight gained. A more detailed study of the difference in the ability of the two groups of lambs to gain weight follows.

Apparent Digestibility Coefficients.—The average apparent digestibility coefficients for the different components of the feed for the 4 male lambs on which these data were obtained are shown in table 4.

There are no significant differences between the average digestibility coefficients of the two groups of any constituent of the feed.

Lamb 72.—Although the average digestibility coefficients for the infected and control lambs failed to demonstrate any significant differences, those for lamb 72, the one lamb dying of trichostrongylosis on which complete nutrition data were obtained, showed significant decreases during the last or ninth nutrition period of the experiment. These data are recorded in table 5.

Although the feces of lamb 72 became soft with the beginning of the eighth nutrition period, no remarkable changes in the apparent digestibility coefficients occurred until the ninth or last nutrition period.

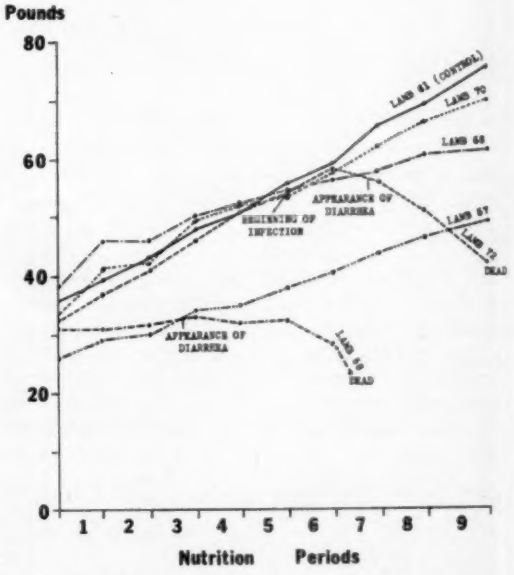

Fig. 2—Weights of infected and control lambs.

During these two periods, lamb 72 did not ingest sufficient feed to maintain itself. (*See* fig. 1). This partial starvation, together with the diarrhea, tended to weaken the lamb so that during the ninth nutrition period it was unable to digest the feed as completely as during the previous portion of the experiment. This fact is shown by the marked decrease in the apparent digestibility coefficients of all constituents of the feed for the ninth period, except those of ether extract (fat) and crude fiber. The decreases in these coefficients were statistically significant.

Data on Nitrogen Metabolism.—The data on the nitrogen metabolism of the 4 experimental lambs, on which these data were obtained, are recorded in table 6.

These data show that lamb 72 had a very large negative nitrogen balance during the time it was infected. This condition was also indicated by the comparatively large percentage of urinary nitrogen excreted. However, there was no change in the percentage of urea or creatinine nitrogen present in the urine from that found in the urine of the uninfected lambs.

Data on Mineral Metabolism.—Data on the mineral metabolism of the 4 male lambs and on the bone analysis of all 6 lambs used in the experiment are given in table 7.

The data in table 7 show that, during the period of infection, lamb 72 had a negative calcium and phosphorus balance. The former was the only negative calcium balance shown by any of the lambs. The latter was more than three times the largest negative phosphorus balance shown by either the control or surviving infected lambs. As in the case of the digestibility coefficients, no radical changes occurred in the mineral metabolism of lamb 72 until the quantity of feed ingested became inadequate to maintain its body weight and its normal physiological processes.

Further evidence of the severe drain on the mineral resources of lambs suffering from clinical trichostrongylosis is given by the data on the composition of the right femurs of the experimental lambs. The ratio of the fat-free organic matter to the ash content of the right femur of lamb 58, one of the ewe lambs which was not put into the metabolism cages, showed a marked decrease as compared to the values obtained from the bone analysis of the remaining lambs. This change in the bone composition of lamb 58 was undoubtedly due to the fact that this animal had suffered from anorexia and diarrhea for fifty-three days, a period eighteen days longer than lamb 72, and sufficiently long to reduce the available mineral matter for metabolic processes to such an extent that the necessary calcium and phosphorus were withdrawn from the bone. The remaining lambs which did not suffer from anorexia or diarrhea, or in which these factors did not operate for a sufficiently long period, did not show changes in bone composition.

Energy Metabolism.—Data on the energy metabolism of the infected and control lambs are recorded in table 8.

TABLE 2—Average Number of Trichostrongyle Eggs (in thousands) Passed per 24-Hour Period by the Experimental Lambs During Nutrition

Lamb No.	Period Number								
	1	2	3	4	5	6	7	8	9
57	0	270	314	900	1,000	1,258	343	300	410
58	0	1,508	1,916	1,093	945	1,234	346	[a]	...
68	712	257	61	1	1	0	2	...	0
70	0	575	1,243	1,572	571	705	179	0	0
72	0	0	0	0	0	0	259	2,123	2,168[b]
61	0	0	0	0	0	0	0	0	0

[a]Lamb died Oct. 4, 1936.
[b]Lamb died Nov. 16, 1935.

TABLE 3—Feed Consumed per Pound of Live Weight Gained by Infected and Control Lambs

Lamb No.	Feeding Period Weeks	I.W. lb.	F.W. lb.	Gain lb.	Total Feed Consumed lb.	Feed Consumed per lb. of Live Wt. Gained lb.
Infected Lambs						
57	18	26	49	23	226.75	9.86
58	6[a]	31	33	2	59.52	29.76
68	18	38	61	23	253.06	11.00
70	18	34	70	36	293.87	8.16
72	2[a]	54	58	4	33.44	8.36
					Average	13.25
Control Lambs						
61	19	36	76	40	306.64	7.67
72	10[b]	32	54	22	132.05	6.00
					Average	6.84

I.W. = Initial Weight. F.W. = Final Weight.

[a]Period from beginning of infection to maximum weight.

[b]Period from beginning of experiment to administration of first dose of larvae.

The data in table 8 parallel those of table 3 and show that, without exception, the infected lambs required more energy than the control lambs to produce a pound of gain.

Incidental and, as yet, unpublished observations on the guanidine content of the blood of a lamb suffering from diarrhea due to trichostrongyle infection, and showing a 100 per cent increase in the quantity of this substance as compared to that present in the blood of a control lamb, will be discussed later in connection with the data on increased energy metabolism.

TABLE 4—Average Digestibility Coefficients of the Different Constituents of the Feed in the Infected and Control Lambs

Lamb No.	Dry Matter	Crude Protein	True Protein	N.P.N. Material	Ether Extract	Crude Fiber	N-free Extract	Organic Matter	Ash
Infected Lambs									
68	69.09	75.24	75.37	74.23	66.47	41.82	80.16	70.30	56.80
70	63.05	65.84	66.16	64.42	63.81	31.83	76.64	64.40	47.89
72[a]	64.45	62.75	65.61	49.60	67.18	40.40	78.91	66.59	39.93
Average	65.53	67.94	69.05	62.75	65.82	38.02	78.57	67.10	48.21
Control Lambs									
61	63.62	63.41	68.64	65.68	62.28	31.05	76.92	64.96	50.53
72[b]	66.31	71.74	73.76	60.88	61.40	30.83	79.36	67.60	51.73
Average	64.97	70.08	71.20	63.28	61.84	30.94	78.14	66.28	51.13

[a]Average digestibility coefficients for nutrition periods 6 to 9 inclusive.
[b]Average digestibility coefficients for nutrition periods 1 to 5 inclusive.

Discussion

The data reported in the present paper show that large infections with the nematode, *T. colubriformis*, apparently reduced the ability of the infected animals to utilize their feed, as shown by their failure to gain weight as efficiently as uninfected control animals. The data, however, do not show any effect on the digestion and absorption of the various components of the ingested feed in the animals not showing clinical symptoms of infection. Similar results were demonstrated by the work on *C. curticei*,[1] and the present findings are explained on the same basis as the previous ones, namely, that the decreased ability to gain weight was probably due to an increase in energy metabolism, due in turn to local nerve irritation and inflammation resulting from the presence of the worms in the intestine.

In the lambs showing clinical symptoms of infection, four additional factors were disclosed: (1) anorexia, (2) diarrhea, (3) retention of guanidine, and (4) prostration, all resulting from the nematode infection and directly affecting the nitrogen and mineral metabolism of the host animal.

The anorexia noted in lambs 58 and 72 brought about a large increase in endogenous nitrogen and mineral metabolism because insufficient feed was ingested to maintain their physiological processes. Thus,

TABLE 5—Apparent Digestibility Coefficients for Lamb 72

Period No.	Dry Matter	Crude Protein	True Protein	N.P.N. Material	Ether Extract	Crude Fiber	N-free Extract	Organic Matter	Ash
1	72.06	73.26	75.94	59.11	64.18	48.13	82.25	73.70	52.31
2	66.74	68.83	72.14	50.07	16.73	32.86	79.33	68.26	48.15
3	63.29	69.86	71.99	58.25	59.10	25.86	77.34	64.60	49.58
4	64.89	74.53	75.03	71.46	61.83	24.70	78.57	65.92	54.15
5	64.55	72.44	73.69	65.49	60.18	22.61	79.31	65.51	54.44
6	63.66	69.53	68.86	73.13	58.96	26.16	78.63	65.24	47.25
7	69.44	72.99	75.25	61.21	66.81	40.06	83.92	71.26	49.68
8	72.15	73.13	71.34	81.13	77.21	59.26	81.92	74.03	49.61
9	52.54	35.36	46.09	—17.09	65.75	36.11	71.17	55.82	13.17
Average	65.48	67.75	70.14	55.86	63.97	35.08	79.12	67.15	46.48
Significant Decreases	±5.94	±12.36	±8.96	±28.88	±5.69	±12.32	±3.61	±5.65	±12.74

these animals were forced to draw upon these materials in muscle and bone, which resulted in a decrease in body weight, a reduction in the ash content of the right femur of lamb 58, and eventually, in a marked debility in both animals.

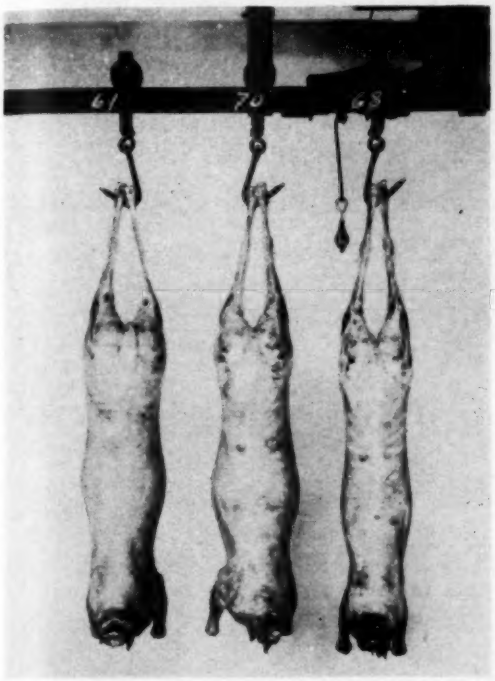

Fig. 3—Top view of carcasses of lamb 61 (control) and lambs 70 and 68, infected with Trichostrongylus colubriformis.

The diarrhea in these 2 lambs also tended to reduce their body weight as well as the quantity of body fluids available for metabolic processes and, at the same time, increased their energy metabolism. The latter was probably due to the increased activity of the intestine and to the retention of guanidine in the blood, a phenomenon occurring wherever severe and prolonged loss of water from the body tissues takes place. The increase in guanidine in the blood of animals has been shown by Staub[5] and Minot[3] and others to give rise to wasteful carbohydrate metabolism.

Prostration, entering into the picture near the fatal termination of trichostrongylosis in lambs 58 and 72, was undoubtedly closely connected with the accumulation of guanidine in the blood, which in relatively large amounts acts as a motor-nerve depressant.

It has been shown earlier in this paper that lamb 72, the one lamb dying of trichostrongylosis on which complete nutrition data were obtained, exhibited toward the close of the experiment a marked decrease in the digestibility coefficients of all but two of the constituents of the feed. It is suggested that this decrease in the digestibility of the feed was indirectly due to the severe loss of water resulting from the prolonged diarrhea which reduced the body

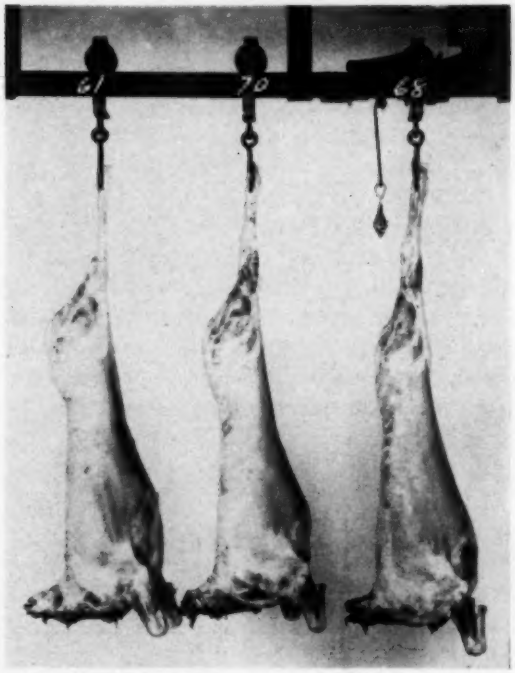

Fig. 4—Side view of carcasses of lamb 61 (control) and lambs 70 and 68, infected with Trichostrongylus colubriformis.

fluids available for the secretion of digestive juices. The reduction in the quantity and quality of these juices, brought about in this way, was probably responsible for the decrease in the percentage of feed digested.

SUMMARY

The nutrition of 4 crossbred Hampshire-Southdown lambs, 3 of which were experimentally infected with the nematode, *Trichostrongylus colubriformis*, was studied in

TABLE 6—Data on the Nitrogen Metabolism of Four Experimental Lambs

Lamb No.	Nitrogen[1] Stored	Urinary[1] Nitrogen	Urea[2] Nitrogen	Creatinine[2] Nitrogen
Infected Lambs				
68	33.13	41.26	56.42	.64
70	23.96	41.95	41.56	1.38
72[3]	—84.72	83.93	56.22	.37
Average	—9.21	55.71	51.40	.80
Control Lambs				
61	26.40	42.04	62.76	.68
72[4]	32.17	39.58	46.09	.61
Average	29.29	40.81	54.43	.65

[1]Percentage of intake. [2]Percentage of urinary nitrogen. [3]Data from last four nutrition periods. [4]Data from first five nutrition periods.

a feeding experiment. Limited observations on 2 additional infected lambs, which could not be conveniently handled in the metabolism cages because they were ewe lambs, were also made.

The results of this experiment failed to confirm the findings of Stewart[6] and of Shearer and Stewart[4] that the worms alone produced a depression of the apparent digestibility coefficients of crude protein and crude fiber, or that they decreased the absorption of calcium and phosphorus. The findings with reference to *T. colubriformis*, therefore, are not in accord with Stewart's theory that parasites elaborate an anti-enzyme in quantities sufficient to interfere with the digestive processes of the host. The results of these authors, however, were in part corroborated in lambs showing clinical symptoms of trichostrongylosis and dying of the disease. In 1 lamb, the apparent digestibility coefficients of all components of the feed, except those of ether extract and crude fiber, were decreased when the vitality of the host animal had been so reduced by starvation and diarrhea that normal digestion and absorption could no longer take place. In another lamb, calcium and phosphorus had been removed from the bone in order that the necessary minerals might be obtained for normal metabolic processes.

TABLE 7—Percentage of Calcium and Phosphorus Stored, and the Ratio of Fat-Free Organic Matter to the Ash Content of the Right Femurs of the Experimental Lambs

Lamb No.	Calcium[1]	Phosphorus[1]	Ratio of Fat-Free Organic Matter to Ash in Right Femur
Infected Lambs			1:
68	23.63	—14.75	1.59
70	5.02	—28.65	1.51
72[3]	—12.32	—86.90	1.45
57[3]	....	....	1.43
58[3]	....	....	1.25
Average	5.44	—43.43	1.45
Control Lambs			
61	12.22	— 6.09	1.49
72[4]	12.02	—22.46	...
Average	12.12	—14.27	1.49

[1]Percentage of intake. [2]For last four nutrition periods. [3]Metabolism data not available because lambs could not be put into metabolism cages. [4]For first five nutrition periods.

TABLE 8—Data on the Energy Metabolism of the Experimental Lambs (in therms)

Lamb No.	Energy Intake	Energy Required for Maintenance (Heat Production)	Energy Required for Growth per Lb. of Live Wt., Gained
Infected Lambs			
57	237.6	118.1	5.2
58	62.4[1]	35.5[1]	10.7[1]
68	265.2	139.7	5.5
70	308.0	139.7	4.7
72	35.0[1]	15.7[1]	4.8[1]
		Average	6.2
Control Lambs			
61	321.3	146.5	4.4
72	138.4[2]	68.5[2]	3.3[2]
		Average	3.8

[1]From beginning of infection to maximum weight. [2]From beginning of experiment to the administration of first dose of larvae.

The energy metabolism of all infected lambs was increased, as was also the number of pounds of feed necessary to produce 100 lb. of gain. The increase in energy metabolism in those cases not showing symptoms of infection may have been due to local nerve irritation and inflammation of the intestine brought about by the presence of the worms. The increase in energy metabolism in those cases showing diarrhea was probably due to the increased intestinal activity and the retention of guani-

dine in the blood, as a result of severe and prolonged water loss.

The presence of *T. colubriformis*, even in considerable numbers, did not produce symptoms of gastrointestinal parasitism or alter the digestibility coefficients of the ingested feed; it did affect the ability of the host to utilize the feed economically. In the one lamb where the nematodes were present in sufficient numbers to produce severe and prolonged diarrhea, they caused a decrease in the digestibility coefficients and absorption of the different constituents of the feed; this was brought about because the vitality of the infected lamb was reduced to the point where the physiological processes, necessary for life, could no longer operate effectively.

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Venezuelan Type of Equine Encephalomyelitis Virus in Trinidad

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THE VENEZUELAN type of equine encephalomyelitis virus is the agent of a severe equine encephalomyelitis which has been occurring in Colombia[1] since 1935 and in Venezuela[2] since 1936. In 1941, it seems to have invaded also the northern coast of Ecuador.[3]

The corresponding virus was isolated in Venezuela by Kubes and Rios in 1938.[4] The comparative immunological studies[5,6] determined this virus as *sui generis*, differing from both the United States eastern and western encephalomyelitis strains. Nor has there been found any specific relation to rabies virus.[7] On the contrary, an immunological identity with the encephalomyelitis virus isolated later in Colombia was demonstrated.[8]

With regard to Trinidad, this island was considered free from the disease until the colony's Department of Agriculture[9] announced on Oct. 2, 1943, the first outbreak of it in the southern part of the island, *i.e.*, in the zone opposite Venezuelan territory. There, 47 cases in all were diagnosed, 30 in the San Francique Pluck-La Fortune area and 17 in the Debe-Penal area. Eight animals survived the infection.

On Oct. 19, 1943, we received through the courtesy of Major Gilyard from the U. S. Army Veterinary Corps, by this time in Trinidad, two samples of material collected in the infected area some days before: material No. 4 from a horse and material No. 5 from a mule, both in the form of brain tissue conserved in a sterile glycerine solution. By intracerebral inoculations of those materials (5% brain-tissue suspension in saline) into white mice and guinea pigs, the presence of a virus was established in both of them. Material No. 5 had an especially rich virus content.

The isolated agent showed the same properties as the Venezuelan encephalomyelitis strain in Swiss white mice, guinea pigs, and developing chick embryos. Swiss white mice, from the second or third passage on, given intracerebrally 0.02 cc. of a 10 per cent brain-tissue suspension in saline, died in three to five days. In guinea pigs inoculated with the same suspension (0.2 cc. intracerebrally), the course of the disease was still more rapid. The same suspension dropped on to the chorioallantoic membrane of 11-day-old chick embryos killed them in fifteen to twenty hours, their bodies showing hemorrhagic infiltrations equal to those produced by the Venezuelan encephalomyelitis virus.

In order to demonstrate the concentration of the virus in the brain tissue of mice and in the chick embryos, the titration was started from a 1 per cent suspension in saline of 7 mouse brains, on the one hand, and of 16 chick embryos on the other, with a view to eliminating possible individual influences. The titration was carried out in white mice by the inoculation of 6 of them with 0.02 cc. of each tenfold dilution. The mouse-brain-tissue suspensions were mortal in 100 per cent from the dilution of 10^{-2} to 10^{-8}. Seventeen per cent of the mice survived the dilution of 10^{-10}. The embryo-cultured virus suspensions had a dilution end-point of 10^{-7} that indicates a virus concentration which is considerably lower. Those results are in perfect accord with the titration of the Venezuelan ecephalomyelitis virus, which has, in the mouse brain, a titer oscillating between 10^{-6} and 10^{-8}, less frequently 10^{-10} to 10^{-12}. In the chick embryo, the average titer corresponds to the dilution of 10^{-7} and is rarely higher or lower.

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The immunological relation between the recently isolated virus from Trinidad and the Venezuelan encephalomyelitis strain virus (1938) was studied in the protection test as follows: Seventy-two Swiss mice were immunized by means of 4 subcutaneous injections of 0.2 cc. of the Venezuelan antiencephalomyelitis vaccine from chick-embryo-cultured virus, one injection given every other day. For many years, the same vaccine has been used with great success in combating encephalomyelitis in Venezuela. Three days after the last vaccination, the animals were divided into 2 groups for the challenge inoculation: The first group received intracerebrally 6 tenfold dilutions from 10^{-1} to 10^{-6} (using 6 mice for each dilution) of a mouse-brain suspension containing Venezuelan ecephalomyelitis virus, and the second group equal dilutions of the virus proceeding from Trinidad. Both viruses were simultaneously titrated intracerebrally in nonimmunized mice.

The vaccinated mice showed a solid protection against both viruses on test. The degree of immunity was more or less equal against both of them, because the mice of each group withstood about 1,000,000 minimal lethal doses.

In summing up our findings, the following conclusions have been drawn:

1) From 2 studied materials of a horse and of a mule which died in Trinidad with clinical manifestations of encephalomyelitis, a neurotropic virus was isolated.

2) Inoculated into mice, guinea pigs, and chick embryos, this virus showed the same properties as the Venezuelan equine encephalomyelitis strain.

3) The vaccine prepared from Venezuelan chick-embryo-cultured virus conferred to mice an equal protection against both viruses, the homologous and that of Trinidad.

4) Therefore, an immunological identity between those two viruses is suspected.

Work on this theme is being continued.

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The Survival of the Infective Larvae of the Common Ruminant Stomach Worm, Haemonchus Contortus, on Outdoor Grass Plots

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FOR EFFECTIVE control of stomach-worm infection in cattle, sheep, and goats by means of pasture rotation, information on the development and survival of the preparasitic stages of this nematode on pastures is essential. The available information on the survival of the infective larvae has been obtained either by using sheep to test the survival of the larvae on pastures or by the direct determination of the survival rate of infective larvae exposed outdoors.

Using sheep as test animals, Ransom (1906) concluded that stomach-worm larvae survived outdoor exposure near Washington, D. C., from October 25 to June 16, while Kammlade, Graham and Boley (1936) concluded that in Illinois the larvae survived from January 15 to June 13, and Boughton and Hardy (1937) determined that the larvae survived twenty-two months in Texas, on the basis of 2 adult stomach worms found in 1 lamb, and 1 worm in another. Baker (1939) found stomach worms in sheep grazed on a pasture in New York State that had been rested a year. Swales (1940) concluded that eggs and larvae did not survive exposure over winter on a pasture in eastern Canada. Kauzal (1936), in a brief progress report without data, stated that a rest of one to two months during continuous dry weather may serve to free a pasture completely of stomach-worm larvae.

By using the direct examination method, Taylor (1938) found that of a mixture of infective "ovine trichostrongyle larvae" exposed outdoors in the fall on grass plots, the majority died within the first few weeks, and about 95 per cent of the original number within two months. Kauzal (1941) exposed infective stomach-worm larvae in early winter on outdoor grass plots, and, after three days, recovered a maximum of about 90 per cent of the original number and after forty-nine days in the open about 20 per cent. Most of the larvae came from the top half inch of soil; few were from the grass washings.

In view of the diversity of the conclusions on the survival period of infective stomach-worm larvae, and of the need for more information, the following experiments, designed to determine the effect of outdoor exposure at various seasons upon the survival of known numbers of infective larvae, were undertaken.

MATERIALS AND METHODS

Outdoor survival of larvae was tested in 60 specially built concrete boxes, each 2 ft. square, arranged in 5 parallel rows of 12 boxes each. The rows were spaced 2 ft. apart and ran from east to west. The boxes, the walls of which extended 18 in. below the soil surface, were first filled with clay soil to 6 in. below ground level and then with a layer of sandy loam up to ground level. The loam was previously heated in a steam box to kill any nematode eggs or larvae that may have been present. The sides of the box, projecting at least 2 in. above ground level, prevented the rain from washing the larvae away. A seed mixture of pasture grasses (blue grass seed, 1 lb.; orchard grass, 0.5 lb.; red top, 0.25 lb.; timothy, 0.3 lb.; and white clover, 0.1 lb.) was sown at the rate of 0.56 oz. in each box at least three months before using the resulting grass plot in an experiment. At the conclusion of an experiment, at least 3 in. of soil were removed from each box to take out any remaining larvae, and replaced with sterilized sandy loam before reseeding for use in later experiments.

Infective larvae were obtained from cultures of feces from rams having experimental

This work was carried out at the United States Department of Agriculture, Beltsville Research Center, Beltsville, Md.

From the Zoölogical Division, Bureau of Animal Industry, Agricultural Research Administration, USDA.

stomach-worm infections. When needed, the larvae were washed from the culture jars, counted by the dilution technique and put on the center of each of the grass plots by pouring the same volume of stirred suspension of larvae through a funnel and then following with a rinse of 500 cc. of water.

The larvae were recovered from the experimental plots by the following procedure. The first 3 plots examined were divided into diagonal half plots with a string. In order to obtain better sampling, all later plots were further subdivided into equal quarters by marking off an outer 4-in. border strip and an inner triangular strip in each half plot, as shown in figure 2. The grass from each subplot was clipped close to the soil and put into separate beakers. The surface soil was then scraped up, and finally, the first and second inch layers of soil were collected separately by means of a tool, similar to a carpenter's marking gauge, developed for measuring vertical layers of soil.

The grass samples were soaked in water for twenty-four hours, squeezed dry and saved. Subsamples of the washings were examined for live stomach-worm larvae under a dissecting microscope. In most cases, five 20 cc. subsamples from each beaker were examined; where the suspension was dense, subsamples of smaller volumes were examined. When live larvae were found, the grass was again soaked for twenty-four hours, the washings examined and the number of larvae present added to those of the first washing.

To determine the number of larvae in the weighed surface scrapings and soil samples, 100 Gm. subsamples from each were put into the Baermann apparatus for forty-eight hours, and the lower 50 to 60 cc. drawn off and examined for larvae. Both live and dead larvae were counted, since previous experiments indicated that very few, if any, larvae killed

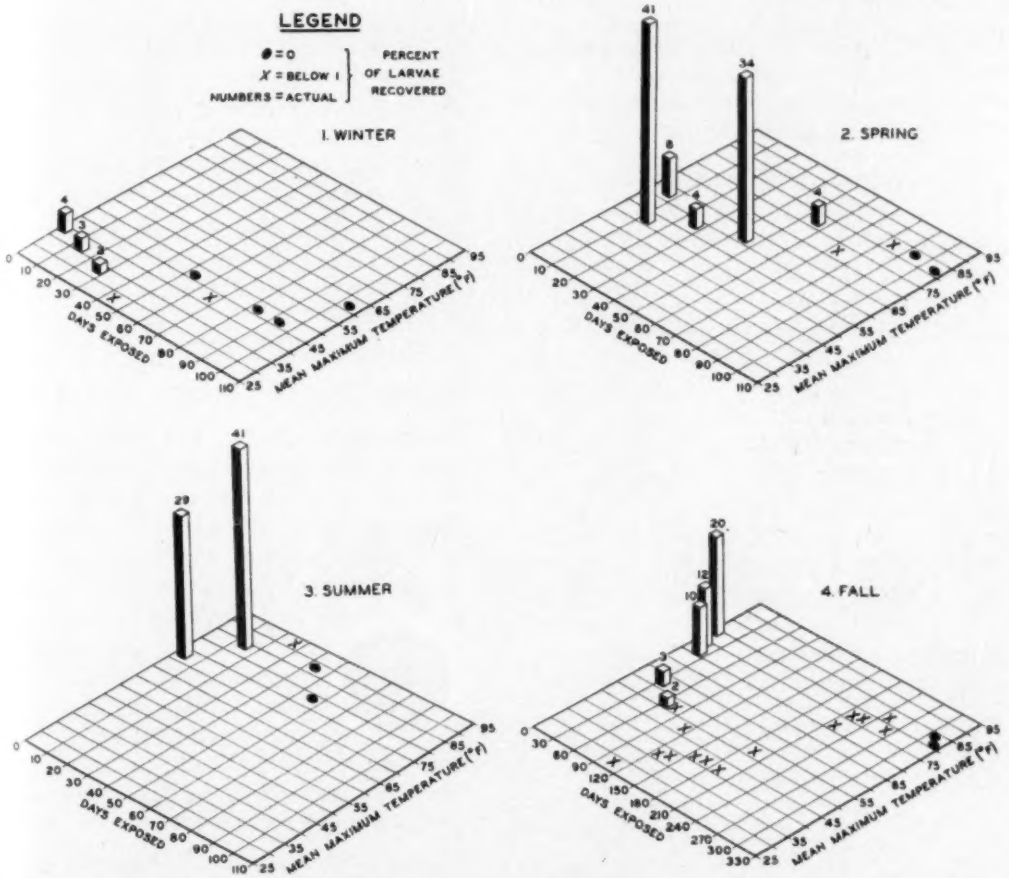

Fig. 1—Percentage of infective Haemonchus contortus larvae, shown by symbols in boxes, recovered after outdoor exposure for indicated periods of time to indicated mean maximum temperatures in 4 experiments.

previously would pass through the screen of the apparatus. From these subsamples, the total number of larvae in the grass or soil samples were readily computed.

Air temperatures were taken with a U-type maximum-minimum thermometer hung on the north side of a tree about 4 ft. above the ground. The thermometer was read daily, in the morning, except Sundays and holidays. Rainfall data were obtained from a gauge located in the Beltsville Research Center, Beltsville, Md., about 1.3 miles from where the experiments were conducted.

EXPERIMENTAL DATA

Four experiments were carried out in 1939-40, beginning in the winter, spring, summer, and fall, respectively. One plot in each of 3 experiments was examined one to two hours after inoculation, and in the fourth experiment, forty-six hours later, to test the effectiveness of the recovery methods. Thereafter, plots selected at random were examined at intervals of fourteen days, except as noted in table 1, where further data on the experiments are presented. The proportion of larvae recovered, given as percentages of the inoculum after indicated exposure periods to the mean maximum air temperatures, is shown in figure 1.

The period and degree of survival of the larvae, as measured by the percentages recovered, varied in the different experiments. In all, there was a more or less rapid decline from the proportion recovered after the first two weeks to less than 1 per cent of the inoculum, and thereafter a persistence at this low level until no more larvae were recovered. This decline to less than 1 per cent recovery occurred after fourteen to twenty-eight days outdoors in the summer, twenty-seven to forty-one days in the winter, forty-two to fifty-six days in the fall, and fifty-six to seventy days in the spring. No larvae were recovered for the first of two consecutive times after forty-two days outdoors in the summer, eighty-three days in the winter, ninety-eight days in the spring and 316 days in the fall. From these data, it appears that the extreme weather conditions of the summer and winter experiments were more unfavorable to survival of the larvae than the conditions of the spring and fall. Of the two weather conditions measured, the amount of rainfall did not bear any direct relationship to the period and degree of survival, but the maximum air temperatures did.

The results of examinations of plots in experiment 4 showed some larvae survived exposure for as long as 266 days. To test whether such larvae as might have survived in other plots of the same series were capable of infecting sheep, a lamb was allowed to graze on 2 of the plots. This lamb, as determined by previous fecal cultures, was free from nematodes, except for *Strongyloides papillosus*. It was allowed to graze for nine days but acquired no infection during sixty-six days of observation. At necropsy, it contained no worms.

HORIZONTAL AND VERTICAL DISTRIBUTION OF LARVAE

Since migration of the larvae might influence their survival, information on their distribution was also obtained during the examination of the plots. This migration was measured by the percentage of the total number of larvae recovered in an experiment which was found in various locations (table 2). To make the results from the first 3 plots of experiment 1 consistent with those of the remainder, the numbers of larvae recovered were allocated to inner and outer quarter plots in proportion to the numbers recovered from later quarter plots in the same experiment.

The small amount of horizontal migration is shown by the very low recoveries from the outer border portions. As for vertical migration, the major portion (67%) of all larvae recovered was found on the grass (43%) or on the surface of the soil (24%). The recovery of 32 per cent of the larvae from the 1-in. layer was due mainly to 2 isolated and unusually high recoveries from the two-week plot in experiment 2, and the two-hour plot in experiment 4. Except for these cases, recoveries were generally low from the 1- and 2-in. layers of soil. In addition to the data in table 2, detailed data for each examination showed that the proportion of larvae recovered

from the 1- and 2-in. layers of soil did not increase with longer exposures.

DISCUSSION

The conclusions of Ransom (1906) on the overwinter survival of stomach-worm larvae on pasture have been circulated so widely that it is worth while to reëxamine the work on which he based his conclusions. In one experiment on a method of raising lambs free of nematodes, Ransom found 1 stomach worm in each of 2 lambs, while the other 11 lambs were free of stomach worms. In another experiment to test the survival of stomach-worm larvae on pasture, newly weaned lambs, raised as in the first experiment, were put on a pasture which had been rested from October, 1905, until June, 1906, for a test period from June to September or October, 1906. On finding numerous stomach worms at necropsy in 4 lambs, Ransom concluded that pastures could not be freed of stomach-worm eggs and larvae after the indicated rest period under climatic conditions similar to those of Washington, D. C. This conclusion is open to question in view of the pos-

TABLE 1—Recovery of Infective Larvae of *Haemonchus contortus* After Outdoor Exposure on Grass Plots to Indicated Temperature and Rainfall Conditions

Experiment (No.)	Date Inoculated	Date Collected	Time Exposed (Days)	Mean Max. Temp.*†	Rainfall† (In.)	No. Larvae Inoculated per Plot	Recovery of Inoculum (%)
1	1939 Dec. 21	1939 Dec. 21	2/24	37.0	....	215,754	4.1
		1940 Jan. 3	13	35.0	0.80		2.8
		Jan. 17	27	34.6	1.38		2.3
		Jan. 31	41	27.8	1.00		0.3
		Feb. 14	55	45.3	0.96		0
		Feb. 28	69	41.6	2.05		0.5
		Mar. 13	83	47.2	1.22		0
		Mar. 27	97	47.5	1.92		0
		Apr. 10	111	61.3	2.96		0
2	1940 Mar. 20	Mar. 20	1/24	63.0	....	103,230	8.2
		Apr. 3	14	54.2	0.46		41.4
		Apr. 17	28	59.1	3.02		4.4
		May 1	42	62.9	2.95		34.2
		May 15	56	76.8	0.62		4.4
		May 29	70	74.6	2.69		0.8
		June 12	84	83.3	0.50		0.5
		June 26	98	82.8	0		0
		July 10	112	81.5	2.26		0
3	July 3	July 5	1 22/24	74.0	1.14	24,800	29.1
		July 17	14	84.3	0.62		40.5
		July 31	28	93.1	1.15		0.4
		Aug. 14	42	87.0	0		0
		Aug. 28	56	79.2	2.99		0
4	Sept. 18	Sept. 18	2/24	79.0	....	306,000	20.4
		Oct. 2	14	74.6	0.88		11.8
		Oct. 16	28	70.0	1.28		10.2
		Oct. 30	42	56.9	2.05		2.5
		Nov. 13	56	58.5	0.40		0.8
		Nov. 27	70	51.5	2.73		2.1
		Dec. 11	84	43.3	1.90		0.3
		Dec. 26	99	29.5	0.86		0.4
		1941 Jan. 8	112	45.8	2.04		0.1
		Jan. 22	126	37.7	0.92		0.3
		Feb. 5	140	38.4	1.41		0.2
		Feb. 19	154	41.7	0.94		0.2
		Mar. 5	168	43.1	0.17		0.02
		Mar. 19	182	43.2	1.21		0.03
		Apr. 2	196	54.6	1.32		0.04
		Apr. 16	210	70.4	1.27		0.09
		Apr. 30	224	76.5	0.44		0.06
		May 14	238	75.5	0.41		0.1
		May 28	252	83.6	0.47		0.01
		June 11	266	78.0	1.95		0.01
		July 31	316	83.7	8.66		0
		Aug. 27	343	82.7	3.35		0

*Mean maximum temperature in degrees F. †Data cover interval from end of previous to end of indicated exposure period.

sibility that the lambs in the pasture experiment may have acquired, as did those in the first experiment, a few stomach worms during the pretest period. In that event, the four or five months test period on pasture during the summer might have favored the intensification of this initial infection, as shown by Stoll (1929) who administered 45 stomach-worm larvae to one of a pair of parasite-free lambs in the spring and then moved both lambs to various plots. By mid-August, the infected lamb showed a daily egg output of 13,600 per Gm. of feces, and its twin 10,600 eggs per Gm. of feces by early September. In view of these possibilities, the infection found in Ransom's lambs cannot be traced conclusively to stomach-worm eggs and larvae that survived over winter on pasture.

Similar objections may be raised against the conclusions of Kammlade, Graham and Boley (1936), who reported stomach-worm eggs in the feces of 1 sheep in each of the 2 test groups before the sheep were put on pasture for a test period of 70 to 126 days. Baker (1939) also kept his test lambs on pasture three months, and noted that 2 control lambs kept indoors during this period showed no eggs in their feces. However, his conclusion that stomach-worm larvae would survive over winter in New York is in contrast with that of Swales (1940) that they would not survive in eastern Canada.

The alternative method of determining the survival of infective stomach-worm larvae outdoors by the direct examination of material containing known numbers of larvae also has its difficulties. Since the numbers of larvae recovered are affected both by the loss of larvae in the recovery process and by the natural death of larvae, it is necessary to consider the effect of recovery losses before estimating the survival rate from the number recovered.

The loss of larvae in recovery, shown by the low percentages recovered shortly after exposure, may be due to incomplete removal of larvae from the grass, to the dispersal of larvae in the soil, or to the variable efficiency of the Baermann isolation apparatus. Despite the stirring and sampling procedures whereby a 100 Gm. Baermann sample was taken from a 3- to 4-kg. layer of soil from each quarter plot, the possibility of missing larvae in so large a mass is obvious. The efficiency of the Baermann apparatus in the recovery of infective stomach-worm larvae, studied by Dinaburg (1942), was shown to vary with the number of larvae put into the apparatus and with the type of substrate. This information, however, cannot be used to correct for this source of loss in the present work because the substrates are not comparable.

Since the losses during recovery cannot be estimated independently, the data presented in this paper, representing a combined measure of the number of larvae which survived outdoor exposure and escaped loss during recovery, can give only a minimum estimate of the proportion surviving outdoors. Nevertheless, the data

TABLE 2—Vertical and Horizontal Distribution of Haemonchus contortus Larvae Recovered From Grass Plots

Location Diagonal Half-plot	Layer	Percentage of Larvae Recovered From Experiment Number 1 Inner	1 Outer	2 Inner	2 Outer	3 Inner	3 Outer	4 Inner	4 Outer	Average* Inner	Average* Outer
S.W.	Grass	13.1	0	4.9	0.4	42.7	0	38.9	0.9	25.7	0.7
	Surface	7.4	4.5	25.6	1.4	0.1	0.01	0.9	0.1	9.6	0.8
	Soil, 1 in.	0.6	0	28.5	0.7	0	0	0.1	0.3	9.7	0.4
	Soil, 2 in.	0.3	0	0.3	0.03	0	0	0.02	0.1	0.1	0.1
N.E.	Grass	27.4	0	5.8	0.4	56.5	0.7	16.4	0.5	16.1	0.5
	Surface	35.4	0	15.9	0.8	0	0.02	8.9	1.6	12.7	1.1
	Soil, 1 in.	10.1	0	14.0	0.3	0	0	28.5	2.5	20.5	1.4
	Soil, 2 in.	1.2	0	0.7	0.2	0	0	0.2	0.04	0.5	0.1
Total Percentage		95.5	4.5	95.7	4.2	99.3	0.73	93.9	6.0	94.9	5.1
Total No. of Larvae Recovered		21,443		96,801		17,361		152,545		288,150	

*Total number of larvae recovered in 4 experiments from given position, divided by total number of all larvae recovered.

show that the majority of larvae disappeared in a relatively short time and that the most unfavorable periods for their survival were summer and winter, while the most favorable were spring and fall.

Although the experiments reported in this paper were concerned primarily with the outdoor survival of stomach-worm larvae, there remains the equally important question of the infectivity of the surviving larvae, which was not dealt with here except for one test during the fall experiment. In this test, the failure of 1 lamb to acquire any adult stomach worms from plots containing infective larvae exposed 266 days over winter suggests that the larvae probably were not numerous enough to be acquired by, or sufficiently viable to infect the lamb.

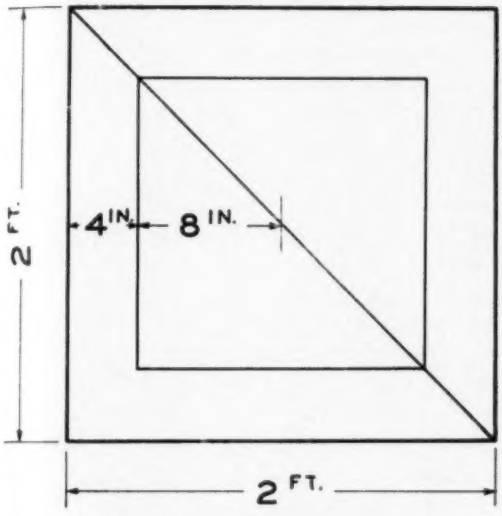

Fig. 2—Subdivision of experimental plots into quarter plots of equal areas.

SUMMARY

1) Known numbers of infective larvae of *Haemonchus contortus* were exposed outdoors on grass plots during four seasons at Beltsville, Md. The plots were examined at intervals of two weeks in most cases.

2) The percentage of larvae recovered fell to about 1 per cent of the inoculum after fourteen to twenty-eight days outdoors in the summer, between twenty-seven to forty-one days in the winter, forty-two to fifty-six days in the fall, and fifty-six to seventy days in the spring.

3) No larvae were recovered after exposures of forty-two, eighty-three, ninety-eight and 316 days in the summer, winter, spring, and fall experiments, respectively.

4) These figures, because of losses during recovery, represent minimum estimates of the survival of the larvae outdoors.

5) A lamb that grazed nine days on 2 plots containing infective larvae which had been exposed for 266 days since fall, failed to become parasitized.

6) Of all larvae recovered in the 4 experiments, 43 per cent were found on the grass, 24.2 per cent on the surface soil, 32 per cent in the first inch of soil, and 0.8 per cent in the second inch. The figure for the first inch is inflated by two exceptionally high recoveries. The horizontal migration of the larvae was comparatively slight.

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Differentiation of Bacillus Anthracis from Nonpathogenic Aërobic Spore-Forming Bacilli

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MANY REPORTS have appeared in the literature on the results of studies of aërobic, spore-bearing bacilli, biologically allied to and closely resembling *Bacillus anthracis*. While these anthrax-like organisms are generally referred to as pseudo-anthrax organisms or anthracoid organisms, various designations have been used by different workers to identify them.

The nonmotile, aërobic, spore-forming organism isolated from soil by Hueppe and Wood in 1889[1] and designated *Bacillus anthracoides*, and the motile, aërobic, spore-bearing organism isolated from Chinese horse hair by Bainbridge in 1903,[2] which he also called *B. anthracoides*, are examples of some of the best known of the early isolations.

Fitch in 1911,[3] reported the isolation of a nonpathogenic organism morphologically and culturally resembling *B. anthracis* from a suspected case of anthrax in a horse. In his studies, he compiled and published a list of 20 such organisms that had been reported by different workers up to that time. Since most of these organisms were motile and only a few were weakly pathogenic, Fitch concluded that the principal difference between the anthrax-like organisms and *B. anthracis* is in their motility and nonpathogenicity. This conclusion is in accord with the views of the author, who pointed out in a previous paper[4] that the most important criterion in the laboratory diagnosis of anthrax is the pathogenicity test.

Siribaed, in 1935,[5] reported the isolation of an anthrax-like organism, which he designated as *Bacillus siamensis* and which he considered to be an apparently new, pathogenic variety of *Bacillus subtilis*.

Heaslip, in 1941.[6] reported the isolation of a motile, spore-bearing bacillus resembling *B. anthracis*, that was lethal for laboratory animals in large doses and occasionally in small doses, and which he designated *Bacillus tropicus*. The organism occurs in man and animals in North Queensland, Australia, and is believed to be the probable cause of a febrile condition in man known as coastal fever. Heaslip suggests the possibility that *B. tropicus* may be a fixed attenuated form (variant) of *B. anthracis*, or that both may be variants of a saprophytic bacillus. In Heaslip's report, 40 cases of anthrax-like infection in man and animals are also cited, 16 of which were regarded clinically as anthrax and 5 as suspected anthrax. However, organisms pathogenic for laboratory animals were isolated from only 6 of the 21 suspected cases of anthrax. He suggested the probability that, in each of the 21 cases mentioned, the organism recovered was a variant of *B. anthracis*.

Ford[7] suggested that *B. anthracoides* reported by some workers was probably *Bacillus cereus*. Clark,[8] who agrees with this opinion, reported evidence to indicate that *B. cereus*, when first isolated, may be weakly pathogenic and that *B. siamensis* and *B. cereus* are culturally indistinguishable, and suggests the probability that some of the anthrax-like bacilli of earlier workers were in reality merely strains of *B. cereus*.

Schweiger, Trainer, and Eveleth, in 1943,[9] reported isolations of *B. subtilis* from actinobacillosis-like lesions in cattle and sheep. The organisms, when first isolated, possessed pathogenic properties but the virulence progressively diminished when cultures were carried on nutrient agar.

Despite the fact that nonpathogenic, aërobic, spore-forming organisms resem-

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bling *B. anthracis* are often encountered in material suspected of containing anthrax and frequently confuse the laboratory diagnosis, little attention has been given in this country to comparative studies of the biochemic reactions and colony formation of *B. anthracis* and anthrax-like organisms.

In the absence of pathogenicity tests, the differentiation of highly attenuated or nonpathogenic strains of *B. anthracis* from organisms closely resembling them by morphology and ordinary cultural tests is sometimes very difficult.

Since the use of the precipitin test, the complement-fixation test, and the ferric chloride chemical test of De Angelis[10] for differentiation of *B. anthracis* from anthrax-like organisms in the absence of pathogenicity tests was not entirely satisfactory in our hands, the following described studies were undertaken to determine whether or not cultural characteristics, reactions on diagnostic mediums, and colony formation would be of any value in distinguishing *B. anthracis* from the common, aërobic, spore-forming organisms resembling it.

Material and Methods

The cultures used in these comparative studies included 53 different strains of *B. anthracis* and 64 different known strains of aërobic, spore-forming organisms.

The collection of anthrax cultures included 23 virulent bovine strains isolated from cases occurring during outbreaks in Virginia, Maryland, North Carolina, South Carolina, Missouri, New Mexico, and South Dakota; 4, virulent, porcine strains isolated from cases of chronic or acute anthrax in swine; 2, virulent, equine strains; 1, virulent, ovine strain; 1, virulent, canine strain; 2, virulent, fly strains; 2, virulent, human strains; 12, attenuated, vaccine strains; and 6, avirulent strains.

The collection of soil cultures tested represented 11 strains of *B. subtilis* obtained from D. F. Eveleth, University of Arkansas; 4 strains of *B. subtilis;* 3 strains of *B. cereus;* and 2 strains of *Bacillus mycoides* obtained from the American Type Culture Collections; 9 strains of *B. subtilis;* 16 strains of *B. cereus;* 6 strains of *B. mycoides* and 1 strain each of *B. siamensis* (Siribaed), *B. tropicus* (Heaslip), *Bacillus simplex, Bacillus carotarum, Bacillus circulans, Bacillus megatherium, Bacillus firmus, Bacillus flexus, Bacillus mesentericus,* and *Bacillus brevis* furnished through the courtesy of N. R. Smith, Bureau of Plant Industry, Soils and Agricultural Engineering, USDA; also 2 strains of *B. mycoides* B. A. I. of recent isolation, and 1 strain of *B. cereus B. A. I.* isolated by the author from a specimen which was suspected of containing anthrax.

The morphology, cultural, biochemic, and pathogenic techniques used in the comparative tests are the same as those commonly used in this laboratory in the routine examination of cultures.

Motility tests were made by microscopic examination of hanging drop preparations from young, broth cultures.

Growth characteristics of the various organisms tested were observed in broth cultures and a macroscopic and microscopic study was made of colony formation on the surface of plain agar plates after 18- to 24-hours incubation.

Hemolysis for all cultures was determined by the tube method, that is, by addition of 1 cc. of a 5 per cent suspension of sheep red blood cells in normal saline to 1 cc. of the broth cultures. Tubes were incubated for two hours at 37.5 C. and held in the icebox over night. A limited number of strains of *B. anthracis* and the anthrax-like organisms were also tested for hemolysis on 5 per cent equine blood-agar plates, 5 per cent ovine blood-agar plates, and 5 per cent bovine blood-agar plates.

The basic, beef-infusion, broth medium used in these experiments was prepared from beef extract and contained 1 per cent peptone (Difco) and 0.5 per cent sodium chloride. The medium was adjusted to a pH of 7.4. The plain, agar medium used for carrying stock cultures and making agar plates for colony study was a clear, transparent medium composed of basic, beef-infusion broth with the addition of 2 per cent agar having a pH of 7.4.

For study of fermentation properties, a total of 14 carbohydrates, 6 alcohols, and 1 glucoside were used.

The medium used for fermentation tests consisted of the basic, infusion broth to which 1 per cent of fermentable material and 1 per cent Andrade's indicator were added. Ten per cent sterile, horse serum was added to dextrose, lactose, and saccharose mediums, and 24 per cent sterile, horse serum to all the other types of sugars. All mediums were sterilized by filtration through Mandler bacterial retaining filters and titrated to pH 7.2.

Readings were made at eighteen, forty-eight, and seventy-two hours, five days, and, in the case of salicin and glycerol, also at seven days.

The medium used for tests to determine the ability of various organisms to reduce methy-

TABLE 1—Biochemic, Cultural, and Biologic Characteristics of Bacillus Anthracis*

No. of Strains	Source or Origin	Pathogenicity	Dextrose	Saccharose	Maltose	Trehalose	Levulose	Xylose	Dextrin	Glycerol	Salicin	Galactose	Lactose	Sorbitol	Inulin	Inositol	Adonitol	Mannite	Mannose	Rhamnose	Arabinose	Raffinose	Dulcite	Indol production	Nitrate reduction
		Group I—Virulent Strains																							
23	Bovine	+ + +	+	+	+	+	±	T	+	±	±	—	—	—	—	T	—	—	—	T	T	—	—	—	+
4	Porcine	+ + +	+	+	+	+	±	T	+	±	±	—	—	—	—	T	—	—	—	T	—	—	—	—	+
1	Ovine	+ + +	+	+	+	+	±	T	+	±	—	—	—	—	—	—	—	—	—	—	—	—	—	—	+
2	Equine	+ + +	+	+	+	+	±	T	+	±	—	—	—	—	—	T	—	—	—	—	—	—	—	—	+
1	Canine	+ + +	+	+	+	+	±	T	+	±	—	—	—	—	—	—	—	—	—	—	—	—	—	—	+
2	Fly	+ + +	+	+	+	+	±	T	+	±	—	—	—	—	—	—	—	—	—	—	—	—	—	—	+
2	Human	+ + +	+	+	+	+	±	T	+	±	±	—	—	—	—	T	—	—	—	T	—	—	—	—	+
		Group II—Vaccine Strains																							
2	No. 1 Spore	+	+	+	+	+	±	V	+	V	±	V	—	—	—	V	—	T	—	—	T	—	—	—	+
6	No. 2 Spore	+ +	+	+	+	+	V	V	+	V	±	V	—	—	—	V	—	T	—	—	T	—	—	—	+
2	No. 3 Spore	+ +	+	+	±	+	V	V	+	V	±	V	—	—	—	V	—	T	—	—	T	—	—	—	+
2	No. 4 Spore	+ +	+	+	+	+	±	V	+	V	±	V	—	—	—	V	—	T	—	—	T	—	—	—	+
		Group III—Avirulent Strains																							
1	Porcine	—	+	+	+	+	±	—	+	±	—	—	—	—	—	T	—	—	—	—	—	—	—	—	+
5	Old Laboratory Strains	—	+	+	+	+	±	T	+	±	±	V	—	—	—	T	—	T	—	T	—	—	—	—	+

*All strains were Gram positive, nonmotile; failed to reduce methylene blue broth in 48 hours or to produce complete hemolysis in 18 hours; slowly liquefied gelatine, slowly acidified, coagulated, reduced and peptonized litmus milk and produced little or no turbidity in broth.

Legend: Pathogenicity: (+ + +) kills guinea pigs in 48 hours or less; (+ +) kills guinea pigs in 72 hours or longer; (+) kills mice but not guinea pigs; (—) fails to kill either mice or guinea pigs.

Carbohydrates: (+) fermentaton; (±) slight fermentation; (±) slight fermentation after 72 hours (variable); (V) may or may not be fermented; (T) trace; (—) no fermentation.

TABLE 2—Biochemic, Cultural, and Biologic Characteristics of Bacillus Anthracis (R. Type) Compared With Those of Type Cultures of Aeröbic, Spore-Bearing Organisms*

TABLE 2—Biochemic, Cultural, and Biologic Characteristics of Bacillus Anthracis (R. Type) Compared With Those of Type Cultures of Aeröbic, Spore-Bearing Organisms*

No. of strains	Organism tested	Gram stain	Motility	Dextrose	Saccharose	Maltose	Trehalose	Levulose	Xylose	Dextrin	Glycerol	Salicin	Galactose	Lactose	Mannite	Mannose	Rhamnose	Arabinose	Raffinose	Dulcite	Indol production	Nitrate reduction	Reduction of methylene blue broth 48 hours	Liquefaction of gelatin	Hemolysis
1	B. anthracis	+	—	+	+	+	+	±	—	+	±	±	—	—	—	—	T	T	—	—	—	+	—	+	—(3)
	Group A. Aerobic, Spore-Bearing Organisms Closely Resembling Anthrax																								
20	B. cereus	+	+ +	+	V	+	+	+	—	+	—	V(2)	—	—	—	+	—	—	—	—	—	+	+	+ + +	+ + +
5	B. mycoides	+	—	+	+	+	+	+	—	+	—	—	—	—	—	—	—	—	—	—	—	+	—	+ +	—
5	B. mycoides dissociated	+	?(1)	+	+	+	+	+	—	+	—	V	—	—	—	—	—	—	—	—	—	+	—	+ +	V
24	B. subtilis	+	+ + +	+	+	V	V	+	—	V	—	V	—	—	V	V	—	V	—	—	—	+	V	+ +	—(3)
1	B. siamensis	+	+	+	±	±	+	+	—	+	—	—	—	—	—	—	—	—	—	—	—	+	+	+ + +	+ +
1	B. tropicus	+	+ +	+	+	±	+	±	—	+	—	±	—	—	—	—	—	—	—	—	—	+	+	+ + +	+ +
	Group B. Aerobic, Spore-Bearing Organisms Not Closely Resembling Anthrax																								
1	B. mesentericus (B. pumilus)	+	+ + +	+	+	—	+	+	—	+	—	+	—	—	±	±	—	±	—	—	—	+	—	+ + +	—
1	B. megatherium	+	+ +	±	±	±	±	±	+	±	—	—	—	—	±	±	—	±	±	—	—	—	—	+	—
1	B. carotarum	+	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	+	—
1	B. simplex	+	+ +	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	+	—	+	—
1	B. circulans	+	+ +	±	±	±	+	±	+	+	—	±	±	±	±	+	—	—	±	—	—	+	—	—	—
1	B. firmus	+	+	±	±	±	±	—	—	±	—	—	±	—	—	—	—	—	—	—	—	+	—	+	—
1	B. flexus	+	+	±	±	±	±	±	—	±	—	±	±	—	±	—	—	—	±	—	—	—	—	+ +	—
1	B. brevis	—	+	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	+	—	+	—

*None of cultures tested fermented sorbitol, inulin, inositol or adonitol All the cultures except *B. anthracis* were nonpathogenic for guinea pigs. All the cultures except *B. anthracis* and *B. mycoides* produced more or less turbidity in broth.

Legend: Motility: (+ + +) actively motile; (+ +) motile; (+) weakly motile; (?) motility questionable; (—) nonmotile.

Carbohydrates: (+) fermentation; (±) slight fermentation; (±) slight fermentation after 72 hours (variable); (V) may or may not be fermented; (T) trace; (—) no fermentation.

Gelatin: (+ + +) rapid liquefaction; (+ +) liquefaction; (+) slow liquefaction; (—) no liquefaction.

Methylene blue broth; (+) partial or complete reduction; (V) may or may not show reduction; (—) no reduction.

Hemolysis: (+ + +) rapid; (+ +) hemolysis; (V) may or may not produce hemolysis; (—) no hemolysis.

Notes: (1) According to Conn. N. Y. Agric. Exper. Sta. Bul. 58, 1917, the motility of *B. mycoides* is in dispute. (2) Most strains tested produced marked acidity in salicin in less than 48 hours. (3) Some strains produce slight hemolysis after 18 hours.

lene blue consisted of 10 cc. of basic, infusion broth to which 3 cc. of a 0.04 per cent methylene blue solution was added.

Indol formation was tested by first growing the cultures from three to five days on Dunham's peptone broth and by the use of the Gore test to detect the formation of indol.

Tests for the reduction of nitrates were made on cultures which had been grown from three to five days in nitrate broth.

Pathogenesis was determined by the subcutaneous inoculation of guinea pigs or mice with 18- to 24-hour, broth cultures. In the case of *B. anthracis*, 0.25 to 0.5 cc. of virulent strains and 1 to 2 cc. of vaccine or attenuated strains were injected into the guinea pigs. Only the vaccine and attenuated strains of *B. anthracis* were tested on mice, which received a dose of 0.25 cc. The other types of aërobic, spore-forming organisms were tested only on guinea pigs, the dose being 2 cc. A postmortem examination was made of all laboratory animals that died; the lesions were noted, and smear preparations and cultures were made from the heart blood, spleen, or other organs for the purpose of diagnosis.

Microscopic study of colony formation and photographs of the colonies under low magnification were made through the bottom of Petri dishes, while microscopic study of the colony margins under high magnification was made in accordance with the method suggested by Hagan,[11] that is, by removing the lid of the Petri dish and placing a flamed cover slip over the colony to be examined.

Stained, anthrax colonies were prepared for microscopic study and photomicrographs by first removing them from the surface of plain agar plates by the so-called impression method, after which they were fixed, mounted, and stained, while stained colonies of other types of bacilli were prepared in accordance with the technique suggested by Dienes.[12]

PROTOCOLS OF STUDIES

Table 1 shows a summary of the biochemic, cultural, and biologic characteristics of 53 different strains of *B. anthracis*.

Table 2 shows a summary of the biochemic, cultural, and biologic characteristics of 64 strains of aërobic, spore-forming bacilli compared with those of *B. anthracis*.

Table 3 shows the comparative fermentation reactions on salicin, reduction properties on methylene blue broth, and hemolytic properties of *B. anthracis* and other types of aërobic, spore-forming bacilli closely resembling it.

Table 4 shows the principal points of difference between strains of virulent R type *B. anthracis* and anthrax-like organisms.

Table 5 shows tests for virulency on stock cultures of *B. anthracis* of different ages.

Plate 1 shows the appearance of different types of anthrax colonies on the surface of plain agar plates.

Plate 2 shows colonies of *B. mycoides* made from a freshly isolated strain, from a stock culture strain, and from a dissociated strain.

Plate 3 shows comparative colony characteristics of *B. anthracis* and other bacilli resembling it.

Plate 4 shows the appearance under high magnification of the border or margins of colonies of *B. anthracis* compared with those of some of the more common, aërobic, spore-forming bacilli resembling it.

Additional information and further elaboration on the tabulated data are given in the following protocols:

Biochemic characteristics of *B. anthracis* and other types of spore-forming, aërobic bacilli are shown in tables 1, 2, 3, and 4. Since these studies were concerned primarily with the differentiation of *B. anthracis* from organisms closely resembling it, with no attempt to place them in a correct taxonomic position, the cultural and colony characteristics of only those organisms under group A (table 2) are discussed and compared with *B. anthracis*. For comparative purposes, however, the biochemic and cultural characteristics of the organisms which do not so closely resemble *B. anthracis* are listed under group B and are also included in table 2.

The action of 53 different strains of *B. anthracis* upon fermentable and other types of diagnostic mediums was compared with that of 64 different known strains of aërobic, spore-forming bacilli. The anthrax strains tested included 35 virulent strains, 12 vaccine strains, and 6 avirulent strains.

FERMENTATION REACTIONS

Fermentation with production of acid but no gas was produced in seventy-two hours or less by all the virulent strains of *B. anthracis* tested in dextrose, saccharose, trehalose, maltose, levulose, and dextrin. Some

Plate I—Comparative appearance of different types of colonies of Bacillus anthracis on surface of plain agar plates (24-hour growth). A, B, C, D, E—natural size; F, I, J, K, L—x6; G, H, M—x10.

strains after five to seven days produced very slight acidity in glycerol and salicin. No definite fermentation occurred in lactose, galactose, xylose, sorbitol, inulin, inositol, adonitol, mannitol, mannose, rhamnose, arabinose, raffinose, and dulcitol (table 1). These reactions in general are in accordance with those reported by Kristensen[13] and Hewlett.[14]

In some of the virulent strains of *B. anthracis* tested, however, a faint pinkish hue reliable criterion in themselves for distinguishing *B. anthracis* from these organisms.

Some strain variability was observed in the different strains of *B. cereus*, *B. subtilis*, and *B. mycoides* in their ability to ferment certain carbohydrates.

The fermentation properties of *B. mesentericus*, *B. simplex*, *B. carotarum*, *B. circulans*, *B. megatherium*, *B. firmus*, and *B. flexus*, listed under group B (table 2) were

TABLE 3—Action of Bacillus Anthracis and Anthrax-Like Organisms on Methylene Blue Broth, Salicin, and a 5 per cent Saline Suspension of Sheep Blood Cells

No. of Strains	Organism	Methylene Blue Broth (.009%) Degree of Reduction in 48 Hours		Salicin Degree of Fermentation in 48 Hours			Sheep Red Blood Cells 5% Suspension Degree of Hemolysis		
		None	Partial or Complete	None	Very Slight	Marked	None	Slight	Marked
37	B. anthracis (virulent)	37	0	37	0	0	33	4	0
20	B. anthracis (isolated direct from guinea pigs)	20	0	20	0	0	18	2	0
1	B. anthracis (vir. smooth strain)	1	0	1	0	0	1	0	0
1	B. anthracis (vir. semismooth strain)	1	0	1	0	0	0	1	0
3	B. anthracis (vir. semirough strain)	3	0	3	0	0	2	1	0
14	B. anthracis (attenuated)	14	0	12	2	0	11	3	0
6	B. anthracis (avirulent)	6	0	6	0	0	6	0	0
24	B. cereus	0	24	6	0	18	0	0	24
1	B. siamensis	0	1	1	0	0	0	0	1
1	B. tropicus	0	1	0	0	1	0	0	1
24	B. subtilis	7	17	11	3	10	21	3	0
5	B. mycoides	5	0	2	1	2	2	1	2
1	B. mesentericus	1	0	0	0	1	1	0	0
1	B. megatherium	1	0	1	0	0	1	0	0
15	Undetermined* (spore-bearing aërobic bacilli)	7	8	3	0	12	7	0	8

*Laboratory contaminants or saprophytic organisms isolated from specimens of animal tissue, bone meal, and soil. All nonpathogenic for laboratory animals; 14 out of 15 cultures motile.

(a possible trace of acidity which persisted from twenty-four to forty-eight hours but which was not definite enough to be considered as very slight acidity) was occasionally noted in inositol, rhamnose, arabinose, and xylose.

Fermentation reactions of the avirulent strains were similar to those of the virulent strains, but vaccine strains showed considerable variation, in some instances attacking xylose, galactose, and inositol, and failing to attack levulose.

These studies revealed that the fermentation reactions of *B. anthracis*, with few exceptions, closely resembled those of *B. mycoides*, *B. subtilis*, *B. cereus*, *B. siamensis*, and *B. tropicus* listed under group A in table 2 and, therefore, do not constitute a sufficiently dissimilar from *B. anthracis* to be of some value in differentiation.

By referring to tables 1, 2, and 3, it will be noted that none of the virulent strains of *B. anthracis* tested produced definite acidity in salicin in twenty-four to seventy-two hours, whereas most strains of *B. cereus* and *B. tropicus* fermented salicin in forty-eight hours or less. For differentiation of *B. anthracis* from these organisms, it therefore appears that salicin is one of the most useful carbohydrates, especially when supplemented with tests on other types of diagnostic mediums.

In special tests with salicin, the following results were obtained with cultures incubated forty-eight hours: 80 out of 82 cultures of *B. anthracis* produced no def-

inite fermentation. The other 2 cultures, which were vaccine strains, produced questionable or very slight fermentation.

Eighteen out of 24 strains of *B. cereus* produced marked fermentation, while the other 6 strains produced no fermentation. *B. tropicus* and *B. mesentericus* produced fermentation, while *B. siamensis* and *B. megatherium* produced no fermentation.

Of 24 different strains of *B. subtilis* tested, 11 were negative, 3 showed slight fermentation, and 10 marked fermentation.

Of 5 different strains of *B. mycoides* tested, 2 were negative, 1 showed slight fermentation, and 2 marked fermentation.

These results seem to indicate that if an anthrax-like organism produces definite acidity in salicin in forty-eight hours or less, it is probably not *B. anthracis*.

In this connection, it is interesting to note that N. R. Smith, Bureau of Plant Industry, Soils and Agricultural Engineering, USDA, in a communication dated June 17, 1943, advised that with the use of ammonium phosfate agar with an indicator, 24 out of 27 different cultures of *B. cereus* and 15 out of 17 cultures of *B. mycoides* produced fermentation of salicin.

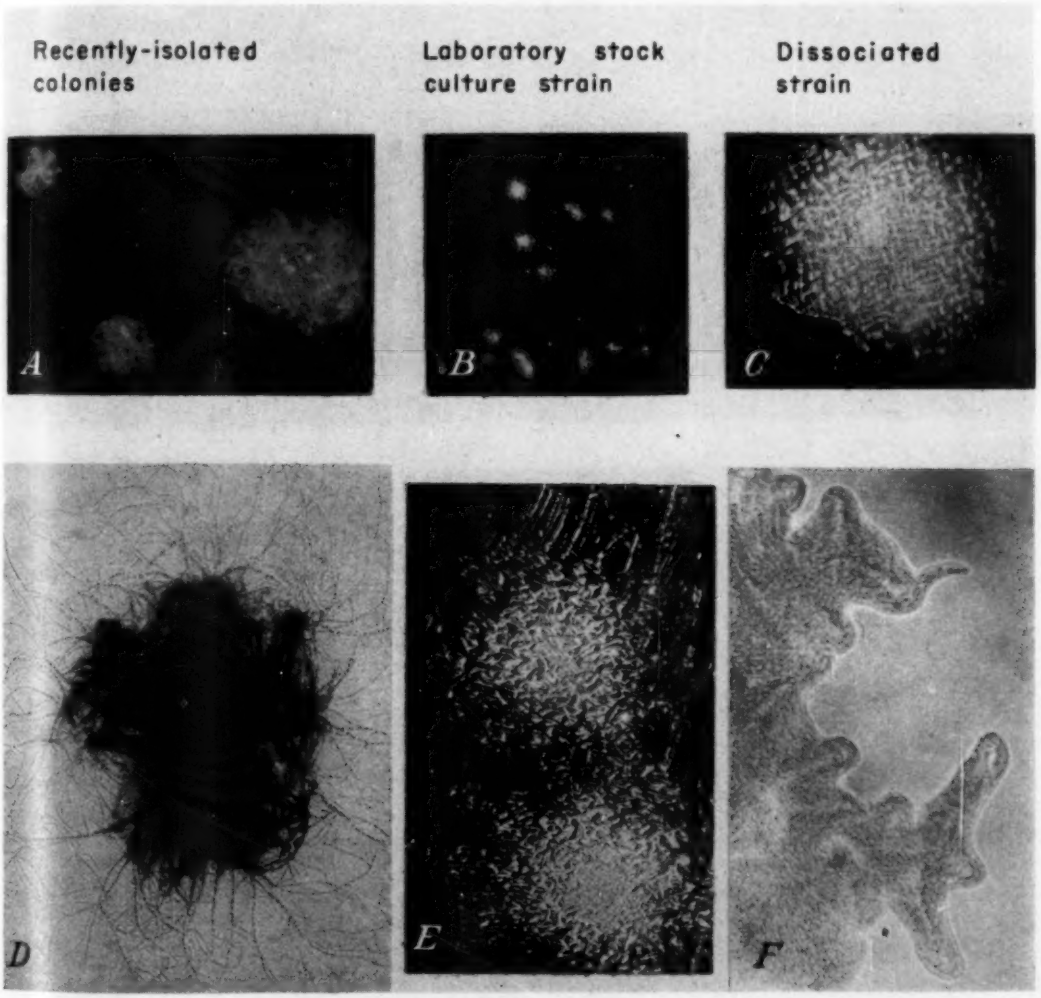

Plate II—Appearance of Bacillus mycoides colonies on surface of plain agar plates (24-hour growth). A and B—natural size; C and E—x6; D—x10; F—margin x75.

BROTH CHARACTERISTICS

In beef-infusion broth, most of the virulent strains of *B. anthracis* tested produced a flocculent growth which settled on the bottom of the tube, forming a sediment which had the appearance of a small ball of cotton, leaving the supernatant broth clear. However, in some virulent strains, a slight turbidity was observed in broth cultures, while a number of the vaccine strains also produced more or less turbidity. In general, strains in which smooth or semi-smooth (dissociated colonies) predominated produced some turbidity.

The growth of *B. mycoides* in broth, which also produces a flocculent sediment with a clear supernatant, is similar to that of *B. anthracis*, but broth cultures made from the other aërobic, spore-forming bacilli tested under group A and commonly confused with *B. anthracis* produced more or less turbidity. Most strains of *B. subtilis* also developed a distinct tough pellicle. Growth characteristics in broth, therefore, are only of limited value in differentiation.

ACTION ON LITMUS MILK

B. anthracis slowly acidifies, coagulates, decolorizes, and finally peptonizes litmus milk. In most of the strains tested, slight acid was first produced in twenty-four to seventy-two hours, curd formation followed in five to seven days, and decolorization and slow digestion required three to four weeks or longer. Some strain variation was noted in the rapidity with which the changes in litmus milk occurred (attenuated strains in particular producing slower reactions than most virulent strains).

B. cereus and *B. siamensis*, in contrast to the action of *B. anthracis*, produced a rapid peptonization of litmus milk with little or no curd formation. Most strains of *B. subtilis* tested usually produced a slight

TABLE 4—Principal Points of Difference between Bacillus Anthracis (R. Type) and Anthrax-Like Organisms*

ORGANISM	PATHOGENICITY FOR GUINEA PIGS	MOTILITY (HANGING DROP METHOD)	REDUCTION OF METHYLENE BLUE BROTH 48-HR.	MARKED ACID IN SALICIN 48 HR.	COMPLETE HEMOLYSIS OF SHEEP BLOOD CELLS (TUBE TEST)	LIQUEFACTION OF GELATINE	ACTION ON LITMUS MILK (PEPTONIZATION)	GENERAL BROTH CHARACTERISTICS	CHARACTERISTICS OF GROWTH ON AGAR
B. anthracis	+	—	—	—	—[2]	SLOW	SLOW	clear, or slightly turbid	texture tenacious comes off medium in threads
B. cereus	—	+ or + +	+ +	+ or —[1]	+ + +	RAPID	RAPID	turbid	texture soft, easily removed from medium
B. siamensis	—	+ +	+ +	—	+ + +	RAPID	RAPID	turbid	do.
B. tropicus	—	+ +	+ +	+	+ +	RAPID	RAPID	turbid	do.
B. mycoides	—	—	—	V	—[2]	SLOW	SLOW	clear	rhizoid growth adheres to and penetrates medium
B. subtilis	—	+ + +	V	V	—[2]	SLOW	SLOW	slightly turbid, then clear; pellicle	dry, wrinkled growth. Adheres to medium
B. mesentericus	—	+ + +	—	+	—	RAPID	SLOW	turbid	texture soft, easily removed from medium

*Characteristics given are those common to most strains examined. [1]Most strains acid positive. [2]Some strains very slight hemolysis.
Explanation of symbols: Methylene blue broth: (+ +) partial or complete reduction; (V) may or may not reduce; (—) no reduction.
Salicin: (+) acid; (V) acid or no acid; (—) no acid.
Hemolysis: (+ +) hemolysis; (+ + +) marked hemolysis; (—) no hemolysis.

alkalinity, decolorization, and slow peptonization with little or no coagulation; *B. mycoides* and *B. mesentericus* slowly decolorized and peptonized litmus milk with little or no coagulation, and *B. tropicus* rapidly acidified, reduced, and peptonized litmus milk. While the reactions in litmus milk may be of help in differentiating *B. anthracis* from *B. cereus*, *B. siamensis*, and *B. tropicus*, as far as other anthrax-like organisms are concerned, they are of limited practical value, because culture tests in this medium must be held for thirty days or longer to properly observe and record the reactions.

Reactions on Gelatin

Stab cultures of *B. anthracis* in 15 per cent gelatin are characterized by a filamentous growth along the line of inoculation, which has the appearance of an inverted pine tree and which is of some value in differentiation from the anthrax-like organisms.

While most of the strains of *B. anthracis* tested liquefied gelatin slowly, there was considerable strain variation in the rapidity of liquefaction. The speed of liquefaction manifested by different strains ranged from four to twenty-one days or longer. A few virulent strains that produced liquefaction in four to five days were encountered, but liquefaction in most virulent strains proceeded slowly.

TABLE 5—Tests on Virulency of Stock Cultures of Bacillus Anthracis*

No. of Cultures Tested	Year Isolated	Pathogenic for Guinea Pigs in Less Than 48 Hours†	Approximate Length of Time Remained Virulent
			(years)
4	1932	4	11
4	1933	4	10
3	1934	3	9
1	1936	1	7
3	1939	3	4
2	1940	2	3
1	1941	1	2
2	1942	2	1

*All cultures maintained on plain agar slants, ph 7.4, stored in refrigerator and transferred every 60 days.
†Dosage 0.25 cc. of 24-hour broth culture subcutaneously.

With vaccine strains in general and avirulent strains, the rate of liquefaction was usually somewhat slower than with virulent strains.

B. cereus, *B. siamensis*, *B. tropicus*, and *B. mesentericus* rapidly liquefied gelatin in from twenty-four to seventy-two hours. *B. subtilis* and *B. mycoides*, on the other hand, liquefied gelatin more rapidly than *B. anthracis* but with less speed than the four organisms first mentioned. The speed of liquefaction of gelatin produced by these organisms is consequently of some value in their differentiation from *B. anthracis*.

Test for Hemolysis (Table 3)

All the strains of *B. anthracis* and the other types of aërobic, spore-bearing organisms examined were tested for hemolysis by means of the tube test, that is, by adding 1 cc. of a 5 per cent saline suspension of sheep red blood cells to an equal amount of 24-hour broth culture of the organism to be tested.

None of the strains of *B. anthracis* tested produced complete hemolysis. Of 62 virulent strains, 54 produced no hemolysis, while the other 8 strains produced only a slight hemolysis. Of 14 vaccine strains, 11 produced no hemolysis; and 3, slight hemolysis. Of 6 avirulent strains, all produced no hemolysis.

All strains of *B. cereus*, *B. siamensis*, and *B. tropicus* tested produced complete hemolysis. Of 24 strains of *B. subtilis*, 21 produced no hemolysis; and 3, slight hemolysis. Of 5 strains of *B. mycoides*, 2 produced hemolysis; 1, slight hemolysis; and 2, no hemolysis. *B. mesentericus* and *B. megatherium* produced no hemolysis.

A limited number of strains of virulent *B. anthracis* and the organisms closely resembling it were also tested on 5 per cent equine blood-agar, 5 per cent bovine blood-agar, and 5 per cent ovine blood-agar with similar results. None of the strains of *B. anthracis* produced definite hemolysis after 18- to 24-hours incubation. A few strains, however, produced a slight hemolysis characterized by a narrow, faint zone after 36-to 48-hours incubation. *B. mesentericus* and *B. megatherium* produced no hemolysis, while 2 out of 5 strains of *B. mycoides* produced slight hemolysis, and 4 strains of *B. subtilis* tested produced no hemolysis.

B. cereus and *B. siamensis* produced a marked hemolysis after 18- to 24-hours incubation, characterized by a fairly wide, clear zone of hemolysis, and *B. tropicus* produced definite but less pronounced hemolysis after the same period of incubation. Tests for hemolysis are, therefore, of considerable value in distinguishing *B. cereus*, *B. siamensis*, and *B. tropicus* from *B. anthracis*.

In this connection, it is interesting to note that Buza[15] reported that 40 virulent and 25 attenuated strains of *B. anthracis* were nonhemolytic for sheep red corpuscles in broth at pH 7.1, whereas 3 different saprophytes, *B. anthracoides*, *Bacillus pseudoanthracis*, and *Bacillus anthracisimilis* were hemolytic.

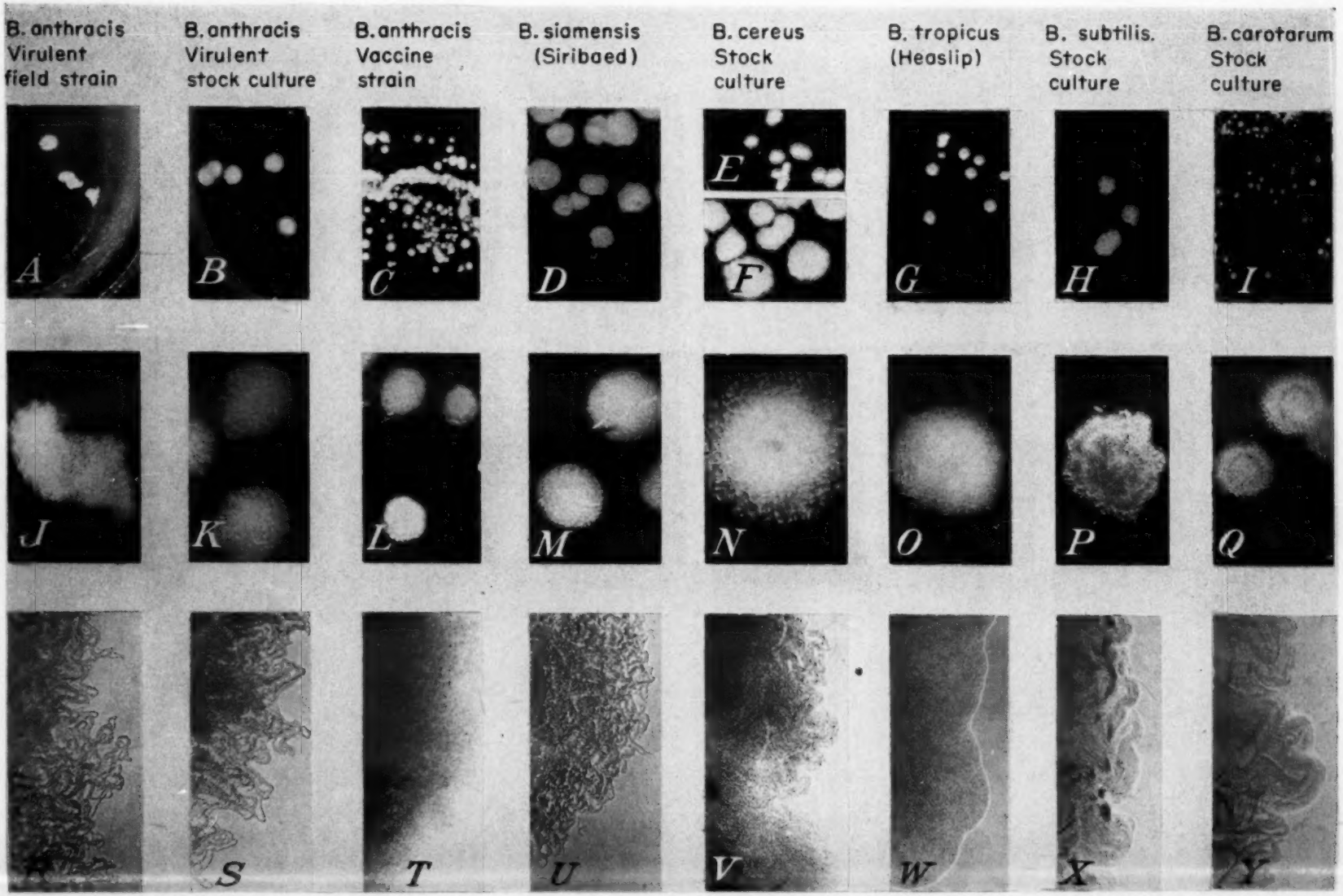

Plate III—Comparative appearance of 24-hour growth of anthrax colonies and anthrax-like colonies on surface of plain agar plates. A, B, C, D, E, F, G, H, I—natural size;

REDUCTION OF METHYLENE BLUE (TABLE 3)

For this test, methylene blue broth, composed of 10 cc. of nutrient broth and 3 cc. of 0.04 per cent methylene blue solution, was used. Eighty-two different strains of *B. anthracis* failed to produce any reduction of methylene blue in forty-eight hours, whereas all the strains of *B. cereus*, *B. siamensis*, and *B. tropicus* tested produced partial or complete reduction in forty-eight hours. Of 24 strains of *B. subtilis*, 17 produced partial or complete reduction, while 7 strains produced no reduction in forty-eight hours. All strains of *B. mycoides*, *B. mesentericus*, and *B. megatherium* tested failed to produce any reduction in forty-eight hours.

A limited number of strains of *B. anthracis* and the anthrax-like organisms were also tested on methylene blue milk with similar results.

Tests for the reduction of methylene blue, like those for hemolysis, therefore, appear to be of especial value in helping to distinguish *B. anthracis* from *B. cereus*, *B. siamensis*, and *B. tropicus*.

These results in general are in accord with those of Murànyi,[16] who reported that methylene blue broth containing 3 to 5 cc. of 0.04 per cent methylene blue solution in 10 cc. of broth was decolorized by saprophytes resembling *B. anthracis* in forty-eight hours, but was unaltered by *B. anthracis*.

PATHOGENESIS

The pathogenic properties of all the virulent strains of *B. anthracis* were tested by injection of 0.25 to 0.5 cc. of a 24-hour, broth culture subcutaneously into guinea pigs. All the 35 virulent strains of *B. anthracis* so tested were fully virulent for guinea pigs, producing death in less than forty-eight hours. *B. anthracis* cultures isolated from No. 2, No. 3, and No. 4 anthrax-spore vaccine when inoculated subcutaneously into guinea pigs in doses of 1 to 2 cc. produced death in seventy-two hours or longer, the virulence and time required to kill depending upon the strength of the vaccine.

B. anthracis cultures isolated from No. 1 anthrax-spore vaccines were nonpathogenic for guinea pigs in doses of 2 cc. subcutaneously, but pathogenic for mice in doses of 0.25 cc. subcutaneously.

The 6 avirulent strains tested were nonpathogenic for both guinea pigs and mice.

All the other spore-bearing organisms tested were nonpathogenic for guinea pigs when 2 cc. of 24-hour, broth cultures were injected subcutaneously.

The history connected with some strains of anthrax-like organisms, such as *B. siamensis*, *B. cereus*, *B. tropicus*, and *B. subtilis*, indicates that when first isolated they were pathogenic, but after being carried on artificial mediums for some time their virulence gradually diminished to a point where they were no longer pathogenic.

On the other hand, *B. anthracis* will maintain its virulence for long periods of time when carried as stock cultures on ordinary types of nutrient agar, when frozen for long periods, or when kept in a dry state at room temperature.

A number of the virulent cultures of *B. anthracis* used in these studies were stock cultures that had been maintained on ordinary mediums from seven to eleven years (table 5).

Recent studies indicate that frozen cultures of *B. anthracis* on serum broth held for a period of eight years at —5 to —10 C., and anthrax spores contained in dried blood on cotton swab specimens held at room temperature for over six years were fully virulent.

The susceptibility of all the guinea pigs that remained well for fifteen days or longer following injection with 2 cc. of the anthrax-like, spore-bearing organisms was tested by injecting each subcutaneously with 0.5 cc. of a 24-hour, broth culture of *B. anthracis*. All the guinea pigs died of anthrax in forty-eight hours or less.

The same results were obtained when guinea pigs that had been previously injected with avirulent strains of *B. anthracis* were exposed to virulent strains, indicating that neither the cultures of anthrax-like, aërobic, spore-bearing organisms or those of avirulent anthrax strains established immunity in the guinea pigs against anthrax.

These studies indicate that pathogenicity constitutes the outstanding point of difference between *B. anthracis* and the anthrax-like organisms. Therefore, when any doubt

Plate III—Comparative appearance of 24-hour growth of anthrax colonies and anthracoid colonies on surface of plain agar plates. A, B, C, D, E, F, G, H, I—natural size

exists as to the identity of cultures, isolated in suspected cases of anthrax, tests for pathogenicity should always be resorted to before a final diagnosis is made.

COLONY ON PLAIN AGAR PLATES AFTER EIGHTEEN TO TWENTY-FOUR HOURS INCUBATION

B. anthracis.—The colonies of virulent field strains of *B. anthracis* isolated direct from animal blood or tissues and growing on the surface of plain agar plates are more or less characteristic. They are the typical rough type, being flat and granular in appearance, with a dull, greyish white surface and an irregular margin. They are small or medium sized and irregular in shape, many of them showing projections or curved tails. The colonies have a tough, stringy consistency. Young colonies on agar plates, on account of this characteristic, can be removed intact from the medium for mounting, fixing, and staining. When transferred with a platinum loop, they tend to come away in threads or strips. This characteristic in the consistency of R type anthrax colony is one chief point of difference between it and anthrax-like colonies. The colonies are opaque and have a cut glass appearance when examined with transmitted light.

Microscopic examination under low magnification shows the colony to be composed of a mass of closely twisted filaments resembling bundles of wavy hair or sheep wool, with a dense center and a somewhat lighter, wide, wavy margin. The open spaces between the bundles or loops of hair-like filaments on the border give the margin its characteristic and somewhat lacy appearance.

Under high magnification the typical hair-locked (medusa-head) appearance may be observed at the margin of the colony. The filaments are revealed as parallel-arranged loops, consisting of closely packed, long chains of bacteria with a tendency to turn back into the mass of the colony and with few, if any, loose ends at the margin. The wavy segmented chains of square-ended bacilli arranged end to end are best observed at the extreme margin (plate IV—fig. A and B).

Colonies made from virulent broth cultures or from rough type, vaccine cultures have the same general appearance as those made direct from specimens of blood or spleen from field cases, but are more or less circular in shape, somewhat more dense, with a wavy irregular margin, and usually show no projections.

The typical rough type, anthrax colony described is sufficiently characteristic to be almost diagnostic (plate I—fig. A, B, G, H, and I; plate III—fig. A, J, R, and B, K. S).

The colonies of dissociated strains and those found in attenuated strains may vary in appearance from the typical rough type (R colony) to the smooth type (S colony). Generally speaking, the greater the variation from the rough type the more difficult it becomes to differentiate by appearance between *B. anthracis* and other aërobic, spore-bearing bacilli, especially those forming colonies composed of curling filaments.

The S or smooth type anthrax colony differs greatly from the R or rough type. It is greyish white with a raised, convex, smooth, glistening surface, spherical in shape, with a homogeneous consistency and with a regular margin. The colony is opaque and of uniform density, with no characteristic margin as observed in the R type. Unlike the rough type colony, which consists mostly of long chains of bacteria, the filaments in smooth colonies are composed mostly of short chains (plate I.—fig. D and K).

In addition to the typical rough and smooth type colonies described, the writer has isolated intermediate types of colonies (semirough and semismooth types) from a number of the commercial anthrax spore vaccines. Plain agar plates seeded from 24- to 48-hour broth cultures prepared from old virulent stock culture strains of *B. anthracis* that have been maintained on plain agar slants from six to eleven years also may show some semirough and semismooth colonies, as well as a predominance of the typical rough colonies, indicating that some dissociation has occurred although cultures remain fully virulent for guinea pigs.

Cultures made from the smooth, semi-smooth, or semirough colonies isolated from the stock culture strains were fully pathogenic for guinea pigs (plate I—fig. C and J, F and M; plate III—fig. C, L, and T).

In this connection, it is interesting to note that Nungester[17] described 6 different variants of the R form, namely, the S or smooth form, RS or intermediate between R and S form, RM or R form with mucoid property, SM or S form with mucoid property, RP or phantom form with R characteristics, and SP or phantom form with S characteristics. He further reported that

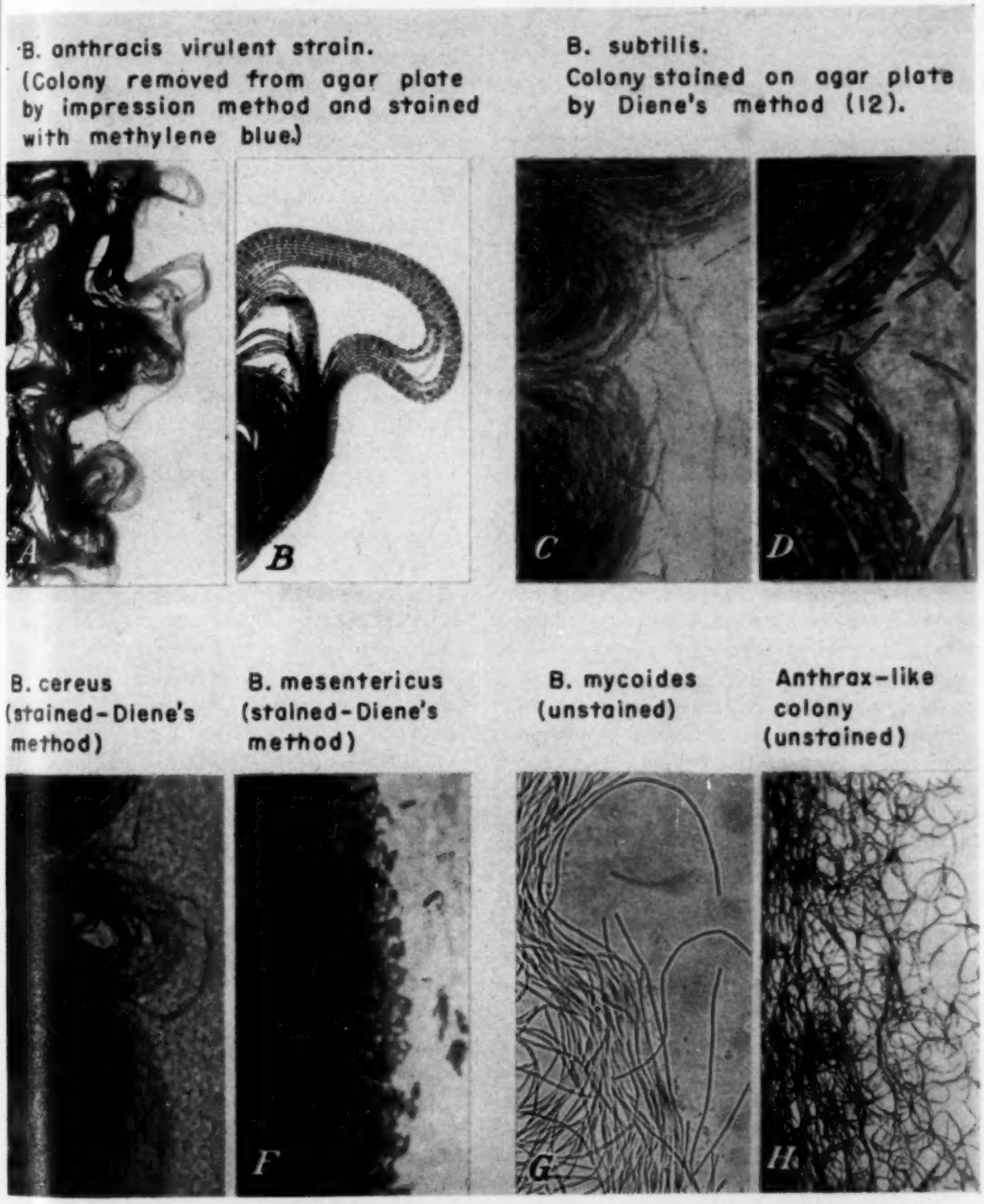

Plate IV—Photomicrographs showing comparative appearance of the margin of an anthrax colony and anthrax-like colonies. A—x145; B—x400; C—x450; D—x1400; E—x300; F—x500; G—x175; H—x145.

the virulence of all forms except the R form, after several transplants on agar, diminished with time.

Another point of considerable interest observed in these studies was the fact that in the more virulent, anthrax-spore vaccines (No. 3 and 4), the small, semismooth or smoothtype colonies predominated, whereas the weaker, anthrax-spore vaccines (No. 1 and 2) showed a predominance of the larger, semirough colonies (plate I—fig. C and J, D and K).

Anthrax-Like Organisms.—Although the colonies of virulent R strains of *B. anthracis* growing on the surface of plain agar plates are more or less characteristic, a number of other types of spore-bearing organisms produce a surface growth on plain agar resembling *B. anthracis*. This is especially true on agar slants when the growth of the colonies is confluent. However, while the appearance of anthrax colonies and anthrax-like colonies may be very similar at first glance, a careful study of the colony and microscopic examination under different powers of magnification will usually show many points of difference. This is particularly evident when the margins of the colonies are examined under fairly high magnification. The loops or bacterial filaments in most of the anthrax-like colonies reveal an irregular arrangement, frequently with interlacing free ends and branching or broken filaments projecting from the border of the colony. The characteristic long, wavy, continuous chains of bacteria in parallel arrangement, with open spaces between loops as are observed in the margin of colonies of *B. anthracis* of R type are usually absent, and instead, more or less dense borders of wide, closely packed bacterial chains are revealed. In actively motile cultures, motility may also be detected in examination of the margin (plate IV).

Colonies of *B. cereus*, *B. siamensis*, *B. tropicus*, *B. mycoides*, *B. subtilis*, and other aërobic, spore-bearing organisms with bacilli arranged in curling filaments may resemble *B. anthracis* when growing on the surface of plain agar plates.

B. cereus produces rapidly growing, medium or large sized, irregular, filamentous or circular-shaped colonies. They have a dry, greyish white, granular surface, a dense center, and somewhat lighter, wavy or irregular border; they resemble the colonies of *B. anthracis*. Unlike *B. anthracis*, the colonies are slightly raised and have a soft instead of tough texture. They can be easily detached by floating off the medium. On microscopic examination, the margin may show loosely arranged, long, wavy filaments with projections and broken loops or closely packed, short filaments with detached bacilli (plates III and IV).

B. siamensis produces rapidly growing, large, irregular, filamentous or circular-shaped colonies, closely resembling *B. cereus*. Their surface has the same granular (ground glass) appearance as *B. cereus* and resembles *B. anthracis*. The texture of the colonies is soft, and they can be easily floated off the medium. The margin, like that of *B. cereus*, may resemble that of *B. anthracis*. In some colonies long, wavy chains of bacilli showing broken loops, free ends, and some detached bacilli may be observed with open spaces between loops (plates III and IV).

B. tropicus produces rapidly growing, medium or large sized, irregular or circular-shaped colonies with a slightly raised, finely granular, greyish white surface, resembling *B. anthracis*. The margin is somewhat wavy, but shows a very close packing of the bacterial filaments. Some colonies show projecting filaments, and motility can be detected in detached organisms at the margin (plate III).

Freshly isolated colonies of *B. mycoides* show a spreading, irregular rhizoid growth. They are flat and granular and greyish white, the surface appearance being similar to that of *B. anthracis*. However, their arborescent growth, with their dense center and filamentous projections, together with the fact that the growth adheres to and penetrates the mediums, readily distinguishes them from colonies of *B. anthracis*. The colonies of dissociated strains of *B. mycoides* do not show a spreading, rhizoid growth. They show fewer filamentous projections at the margin, and the colonies of

some strains examined closely resemble *B. cereus* (plate II).

The colonies of *B. subtilis* may show considerable variation in appearance. Most strains examined produced rapidly growing, irregular or circular-shaped colonies of medium or large size with a dense center and lighter, irregular border. The surface may be slightly raised and granular or slightly wrinkled and membranous. The color may range from a greyish white to a yellowish white. Some colonies, especially those with a dry, granular, greyish surface, resemble *B. anthracis*. The growth, like that of *B. mycoides*, usually adheres to the medium. Microscopic examinnation of the margin may show long or short interlacing chains of bacilli with broken loops and many projecting filaments. Detached bacilli and motility can also be detected at the extreme margin. Some strains show close packing of filaments at border, while others show an arrangement of bacterial filaments at the margin similar to that observed in colonies of *B. anthracis* (plate III and IV).

B. mesentericus produces small, circular, slightly raised, dewdrop-like colonies with finely granular surface and are of a bluish-grey color. The texture of the colonies is soft, and they can easily be floated off mediums. The border of the colony may be finely serrated, and microscopic examination of the margin shows closely packed bacilli singly or in short chains, with numerous detached organisms. Motility can also be observed at the extreme margin (plate IV).

The colonies of *B. carotarum, B. simplex,* and *B. flexus* are somewhat similar and resemble colonies of attenuated strains of *B. anthracis*. They are small and circular, of uniform density, with a raised, finely granular surface and an undulating, curled margin.

The colonies of *B. megatherium, B. circulans, B. firmus,* and *B. brevis* do not resemble those of *B. anthracis*, and, therefore, are not described.

SUMMARY AND CONCLUSIONS

1) Comparative studies were made of the cultural, biochemic, and pathogenic characteristics of 53 strains of *Bacillus anthracis* and 64 strains of aërobic, spore-bearing organisms, some of which closely resemble *B. anthracis*.

2) The results of these studies are given and are also summarized in tabular form.

3) The principal points of difference between *B. anthracis* and the spore-forming aërobes resembling it are pointed out.

4) Colony characteristics of *B. anthracis* and the anthrax-like organisms are described and illustrated.

5) The results of these studies indicate:

A) That the fermentation reactions of *B. anthracis* with few exceptions closely resemble those of anthrax-like organisms such as *Bacillus cereus, Bacillus siamensis, Bacillus subtilis, Bacillus tropicus,* and *Bacillus mycoides,* and therefore do not constitute a reliable criterion in themselves for distinguishing *B. anthracis* from these organisms.

B) That pathogenicity constitutes the principal point of difference between typical strains of *B. anthracis* and those of anthrax-like organisms.

C) That stock cultures of *B. anthracis* maintained on plain agar for as long as eleven years remained fully virulent for guinea pigs when injected in 0.25 cc. doses, whereas cultures of all the anthrax-like organisms tested were nonpathogenic for guinea pigs in doses of eight times that amount.

D) That tests for pathogenicity should always be resorted to when any doubt exists as to the identity of organisms isolated in suspected cases of anthrax.

E) That in the absence of tests for pathogenicity, tests for (1) motility, (2) reduction of methylene blue, (3) hemolysis, (4) fermentation of salicin, (5) rapidity of gelatin liquefaction, (6) action on litmus milk, and (7) colony characteristics, when considered as a whole, will materially assist in the differentiation of *B. anthracis* from the common, aërobic, spore-forming bacilli closely resembling it.

F) That an organism that produces marked acidity in salicin or reduces methylene blue broth within forty-eight hours

and produces rapid and marked hemolysis is not *B. anthracis*.

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The Occurrence of Streptococcus Agalactiae in Colostrum of Heifers under Natural Conditions and Following Artificial Exposure

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WHETHER *Streptococcus agalactiae* can invade the udders of heifers and remain there until lactation begins is a matter of importance in any consideration of this form of mastitis. In the past, control of the disease has consisted largely of the use of sanitation and segregation or elimination of infected cows. Since *Str. agalactiae* was considered an obligate parasite occurring only in the bovine udder and transmitted during milking, eradication of the infection could be accomplished with reasonable success when strict attention was paid to detail. More recently, the development of therapeutic agents has been of assistance in eradication of *Str. agalactiae* infection. Use of these products, however, has by no means obviated the previous practices nor the replacement of diseased cows with healthy ones. First-calf heifers purchased in the late stage of gestation have usually been considered an excellent source for the latter animals. However, some question has arisen from time to time as to the actual freedom of the udders of heifers from infection with *Str. agalactiae*. Because of the common tendency of calves to suck one another after feeding, the possibility of the udder becoming infected during calfhood, when milk containing *Str. agalactiae* formed part of the diet, appeared reasonable. This phase of the problem has, therefore, been the subject of some investigation.

In his article on the presence of *Str. agalactiae* in the udders of heifers, Schalm[1] has dealt with the literature to the end of 1941. Since then, Mattick, Shattock, and Jacob[2] have found these streptococci in 4.5 per cent of first-calf heifers within ten to twenty days after calving and before contact with the milking herd. In a recent report, Van Rensburg[3] states that streptococci appear more frequently in the udders of heifers from herds with a high incidence of infection than from those with little infection. Some years ago, Klimmer and Haupt[4] met difficulty in eradicating *Str. agalactiae* from a badly infected herd. This experience, considered together with a report by Hucker,[5] caused them to question the freedom of the udders of first-calf heifers from *Str. agalactiae*. Minett[6] also suggests the possibility of this calfhood infection from his own efforts to control the disease and from the reports of others. Illustrative of this contention is the report of Munch-Petersen, Murnane, and Bull.[7] These authors assembled a herd of 30 first-calf heifers before parturition and maintained them on premises formerly used for dairy purposes, but where there were no adult cattle at the time. During the first lactation period, *Str. agalactiae* was found in the udders of 43 per cent of these animals on one or more occasions. From these results, the authors feel that great difficulty may be found in selecting heifers not carrying this species of streptococcus. Differing materially are the results of Hastings and Beach,[8] who bought heifers as yearlings and kept them segregated from other cattle during the observation period. Streptococci were never found in the milk of any of these animals during the first lactation period. Some results to which reference is made later in this paper are in agreement with the findings of these authors.

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That the udders of heifers can be infected with *Str. agalactiae* by sucking during calfhood is shown in Schalm's[1] recent report. He found that organism at the time of parturition in 5.7 per cent of heifers that had been fed raw milk from an infected herd and, as calves, had sucked one another indiscriminately. On the other hand, calves in another infected herd that had received pasteurized milk and had been restrained from sucking, were free of *Str. agalactiae* at the time of parturition. In a controlled experiment, he fed infected milk to 4 calves over a period of seven months, during which time the calves were permitted to suck one another. At parturition, 1 heifer was shedding the organism in large numbers from 2 quarters. He concluded that when this species is implanted in the udder during calfhood, it may persist until parturition and be shed in the milk.

Some years ago, one of us (W. T. M.)[9] reported on the incidence and types of streptococci in first-calf heifers at parturition. The present report is in part an extension of the previous one, in that additional heifers have been examined for the presence of streptococci. Some of these animals were observed at regular intervals for several lactation periods to determine whether *Str. agalactiae* might appear subsequently. The second part of this work deals with the results of artificial exposure of virgin heifers with infected milk and diluted broth cultures of *Str. agalactiae* by way of the teat canal.

THE ANIMALS

Of the 77 animals examined bacteriologically for streptococci in the colostrum, 45 were of the same herd that furnished the material included in the earlier report. This herd, reviewed briefly, had an incidence of streptococcal infection, largely *Str. agalactiae*, of between 40 and 50 per cent. The calves were removed from the dams immediately after parturition and grouped together in large box stalls according to age. Unpasteurized milk from the herd was fed as part of the diet and no attempt was made to restrain the calves from sucking. A sample of colostrum was collected from each quarter before the calf could nurse, or as soon as possible after parturition.

The other 32 animals were purchased as first-calf heifers as close to parturition as possible. They were obtained from farms in nearby Maryland and Virginia. A few had been imported from Wisconsin into these states. At the Animal Disease Station, the heifers were kept in a barn and paddock that had never been used by cattle. Samples of colostrum were obtained from the udder before the calf nursed and in a few cases, when possible, preparturition quarter samples were collected.

The 5 heifers used for artificial exposure were the progeny of some of the cows purchased as first-calf heifers. As calves, they were fed milk from the station herd and stabled together without any attempt to restrain them from sucking one another. However, since *Str. agalactiae* was not present in this herd at the time, there was no opportunity for the calves to become infected with this species. When the heifers were about a year old, 3 quarters of each one were exposed with *Str. agalactiae* (*See* table 1). They were bred shortly after exposure, but were brought into the barn only a month or two before parturition. At this time, no cases of *Str. agalactiae* infection existed in any of the older cows in the herd.

EXPOSURE

After the teats had been thoroughly disinfected, 0.1 cc. of milk or diluted broth culture was injected into the quarter by way of the teat canal with a sterile syringe and small gauge needle with a blunt point. No difficulty arose in introducing the material into the teat and it was placed well up in the cistern.

The culture, 13C-2, used for exposure of the right hind quarter of each heifer, was typical of *Str. agalactiae* on differential mediums and belonged to Lancefield's serological group B. However, it produced marked beta hemolysis on horse-blood agar, and the colonies had a slight brown pigment. In addition, a golden brown sediment occurred in trehalose broth. Brown coloration of the sediment of this medium has been characteristic of the several strains of

this type isolated at this station. According to Brown,[10] the culture is a double beta zone streptococcus and belongs in the species described as *Streptococcus mastitidis* by Frost and Engelbrecht.[11] The culture was selected primarily because of its ease of identification on blood agar. It was isolated from a case of acute mastitis several years ago. Before it was used, the culture was transferred rapidly for several days to insure active growth, and then an eighteen-hour culture was diluted to give a small number of organisms for injection. At the same time, various dilutions were plated in blood agar for the bacterial count.

The milk used for exposure was obtained from 2 cows that had been artificially exposed. The left hind quarter of cow 2491 was infected with a typical strain of *Str. agalactiae*. Chronic mastitis had been present for about eight months and had persisted through the dry period. Culture 13C-2 had been used to infect 2 quarters of cow 2513 several months before these heifers were exposed, and the infection was established without difficulty. Several weeks later, a third quarter became infected as a result of natural exposure and developed chronic mastitis. Milk from this quarter was used for exposure material. Blood-agar plates of both samples were made on the day of injection for the bacteria count.

BACTERIOLOGICAL METHODS

The samples of preparturition secretion, colostrum, and milk were incubated overnight at 37 C. After incubation, a loopful of the sample was spread on a blood-agar plate and a second one on a glass slide for microscopic examination. The blood-agar

TABLE I—The Results of Bacteriological Examination of Samples of Secretion from Quarters of First-Calf Heifers Injected with Streptococcus Agalactiae before Breeding

Heifer No.	Quarter*	Date of Injection	Kind of Inoculum	Number of Streptococci	Calving Date	Results of Microscopic and Bacteriologic Tests			
						Preparturition Secretion	Colostrum	Milk	
2516 born 12-22-40	RF	1-5-42	Culture (13C-2)†	144,000	2-10-43	Negative (9 days)‡	Negative	Negative (5 days)‡	Negative (1 month)‡
	RF	3-12-42	Milk (2513A)	2,560					
	LF	1-5-42	Milk (2491D)	670		Negative (9 days)	*Str. uberis*	Negative (5 days)	Negative (1 month)
	LF	3-12-42	Milk (2491D)	1,000					
	RH	3-12-42	Culture (13C-2)	2,800		Negative (9 days)	*Str. agalactiae* (13C-2)	Negative (5 days)	Negative (1 month)
2521 born 2-13-41	RF	3-12-42	Milk (2513A)	2,560	2-13-43	Negative (12 days)	Strep. atypical	Negative (2 days)	Negative (1 month)
	LF	3-12-42	Milk (2491D)	1,000		Negative (12 days)	Negative	Negative (2 days)	Negative (1 month)
	RH	3-12-42	Culture (13C-2)	2,800		Negative (12 days)	Negative	Negative (2 days)	Negative (1 month)
2523 born 1-19-41	RF	3-12-42	Milk (2513A)	2,560	12-17-42	No sample	Negative	Negative (2 months)	Negative (3 months)
	LF	3-12-42	Milk (2491D)	1,000		No sample	*Str. uberis*	Negative (2 months)	Negative (3 months)
	RH	3-12-42	Culture (13C-2)	2,800		No sample	*Str. agalactiae* (13C-2)	Negative (2 months)	Negative (3 months)
2551 born 1-13-41	RF	3-12-42	Milk (2513A)	2,560	1-4-43	Strep. (M) (12 days)	Strep. (M)	*Str. uberis* (14 days)	Negative (2 months)
	LF	3-12-42	Milk (2491D)	1,000		Negative (12 days)	*Str. uberis*	Strep. atyp. (14 days)	Negative (2 months)
	RH	3-12-42	Culture (13C-2)	2,800		*Str. uberis* (3 days)	*Str. uberis*	Negative (14 days)	Negative (2 months)
2573 born 4-10-41	RF	3-12-42	Milk (2513A)	2,560	12-25-42	Negative (6 days)	Negative	Negative (3 months)	
	LF	3-12-42	Milk (2491D)	1,000		Negative (6 days)	Negative	Negative (3 months)	
	RH	3-12-42	Culture (13C-2)	2,800		Negative (6 days)	Negative	Negative (3 months)	

*RF = right front quarter; LF = left front; RH = right hind; LH = left hind. †Number of culture of cow. ‡Time before or after calving. (M) = Microscopic examination only.

plates were examined after forty-eight hours incubation at 37 C., and colonies suspected of being streptococci were picked in serum bouillon. When these organisms were observed microscopically (but did not appear on the blood-agar plate), a second plate was prepared in a further attempt to isolate them. Pure cultures were tested in the differential mediums customarily used for the identification of streptococci.

RESULTS

The incidence of *Str. agalactiae* in the colostrum of the 77 heifers was somewhat lower than that of the previous report.[9] This species occurred in only 1 animal, or 1.3 per cent, although streptococci of various kinds were present in samples from 24 (31.2%) of the heifers either on blood-agar plates or microscopically. In 6 of the 24 animals, streptococci were observed in the incubated milk but could not be found on blood-agar plates. There is, of course, the possibility that some of these were *Str. agalactiae*, but the probability is small for, as a rule, *Str. agalactiae* grows readily on blood agar. Of the cultures that were isolated, 2 were identified as *Streptococcus uberis* and 3 as *Streptococcus dysgalactiae*. The remainder, no doubt present as chance contaminants, differed culturally from each other. Streptococci occurred in 12 animals that were examined a second time within a month. Of these, streptococci had been found in 6 at the first examination, but the same types were present in only 3 of them.

At this time, *Str. agalactiae* had disappeared from the heifer originally carrying it in the colostrum, but 2 heifers, free previously, had acquired the infection. In the interim, all 3 animals had been included in the adult milking herd with a high percentage of streptococcal infection. *Str. agalactiae* was never observed in any of the cows in the herd maintained at the Animal Disease Station in spite of the fact that some of them have been retained and examined for as many as five lactation periods. On the other hand, all 3 cases of *Str. dysgalactiae* infection occurred in the latter herd. In each instance, this species was found in the preparturition secretion from the 4 quarters of each animal as well as in the colostrum. One quarter continued to harbor *Str. dysgalactiae* as well as *Staphylococcus aureus* for almost the entire lactation period. The presence of this species of streptococcus in the udder secretion of preparturient heifers has also been noted by Palmer, Kakavas, and Hay.[12]

The results of artificial exposure of 15 quarters of 5 virgin heifers with milk containing *Str. agalactiae* and diluted bouillon culture of the same species appear in table 1. Streptococci were found in the secretion of 2 of 12 quarters examined before parturition. Although they were observed microscopically, streptococci were never isolated from 1 quarter. From the second quarter, *Str. uberis* was recovered. At the time of calving, 8 of the 15 samples of colostrum contained streptococci. Two of the cultures were identified as *Str. agalactiae*, 4 as *Str. uberis*, and 1 was atypical in differential mediums. No streptococci could be isolated from the last sample, although they were present on microscopic examination. The cultures recovered from the right hind quarters of numbers 2516 and 2523 were identical culturally with the strain of *Str. agalactiae* (*mastitidis*) with which they were exposed. It is of interest to note that both quarters received diluted bouillon culture. All of the exposed quarters were free of streptococci of all kinds within one to three months after parturition.

DISCUSSION

A survey of the literature dealing with *Str. agalactiae* in the udders of heifers prior to first calving reveals a fair degree of agreement on this point. In practically all the reports that have appeared so far, a small number of these animals have been found to be infected at this time. A much higher percentage of heifers has been observed to have streptococci in the colostrum, but most of these types are not associated with bovine mastitis. The findings reported herein are in accord with these results. That an occasional heifer freshens with *Str. agalactiae* seems to be fairly well established. Consequently, it appears logical to assume that if such an animal was in-

troduced into a herd free of *Str. agalactiae*, there would be considerable danger of infecting the other animals.

This point has been brought out by Schalm[1] in connection with 1 heifer that was infected during calfhood and continued to shed *Str. agalactiae* in the milk after calving. The results of the artificial exposure of heifers with milk and cultures reported here appear to support Schalm's[1] findings that *Str. agalactiae* can maintain itself for some time in the nonfunctioning udder. However, the infection failed to persist in either affected quarter. Klein, Klechner, and Schiedy[12] also reported having found *Str. agalactiae* in 7 first-calf heifers some months prior to parturition, but at the time of calving streptococci were not observed in the colostrum. From a consideration of all these results, it would seem that in spite of the infection to which they may be exposed, the udders of heifers are not very susceptible to invasion by *Str. agalactiae*. Furthermore, if these organisms remain in the udder to the time of calving, they may not persist into the lactation period in many animals.

SUMMARY

In continuation of an earlier investigation, samples of colostrum from 77 first-calf heifers were examined bacteriologically for the presence of *Streptococcus agalactiae*. The colostrum was collected before the calf had an opportunity to nurse, or as soon after calving as possible. Forty-five of these heifers were raised in a herd with a high incidence of streptococcal mastitis, largely *Str. agalactiae*. The other 32 animals were purchased from a number of farms and assembled at the Animal Disease Station before calving. They were kept in quarters that had never housed cattle, so far as is known. Natural infection with *Str. agalactiae* has never been found in any of the animals in the latter group.

Str. agalactiae was found in only 1 (1.3%) of the 77 heifers. This animal was a member of the infected herd.

Three quarters of each of the 5 heifers were exposed with *Str. agalactiae* by way of the teat canal. They were exposed at approximately 1 year of age and were bred shortly afterwards. Two quarters were exposed with milk infected with *Str. agalactiae* and the remaining quarter with a diluted bouillon culture of *Str. agalactiae*.

Only 2 of the 15 quarters thus exposed were found to have *Str. agalactiae* in the colostrum at the time of parturition. Preparturition secretion could be obtained from only 1 of these 2 quarters, and the organism was not found in it. Repeated examinations of the milk for from one to three months failed to reveal this species in any of the quarters.

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Two New Enterotome Devices

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IN MAKING parasitological examinations of the digestive tracts of cattle, it is imperative that the time required for preparing the intestines for examination be reduced as much as possible so that selected parasitized areas can be excised and placed in fixatives before postmortem changes occur. Using enterotome scissors for slitting a bovine intestine 100 to 175 feet long is time-consuming and laborious.

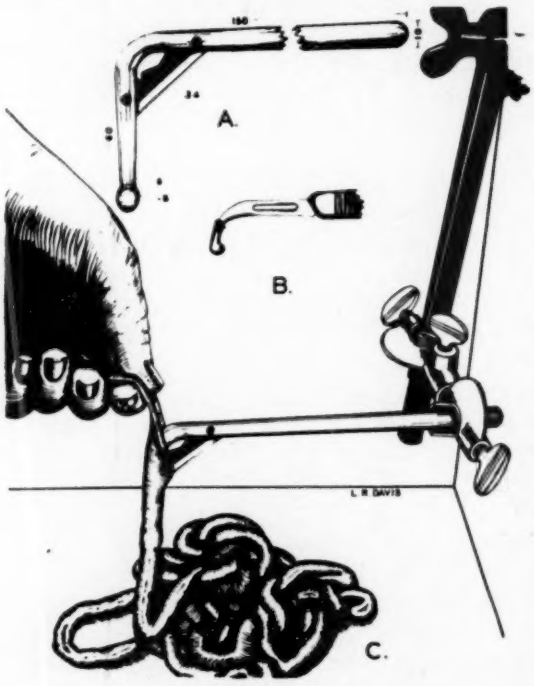

Fig. 1—Enterotome devices: A. dimensions, in millimeters, of larger knife; B. simpler knife for smaller animals; C. larger knife being used in sink.

An enterotome knife[1] has been devised in which half of a double-edged razor blade forms the cutting edge. This instrument makes it possible to slit an intestine longitudinally in one and one-half minutes. The intestine is opened in a straight line on the side opposite the attachment of the mesentery. The instrument (fig. A) was made from a 10 mm. by 250 mm. stainless steel rod. The rod was machined on a lathe until it tapered to a diameter of 6 mm. near one end, leaving a knob 8 mm. in diameter at the end of the knife. The rod was bent at a point 60 mm. from the smaller end to form an angle of 120 degrees. A longitudinal slot, 1 mm. wide by 6 mm. deep, was cut on the inside surface of the angle, extending approximately 35 mm. from the angle toward each end. The rod was then bent further to form a 90 degree angle. A hole penetrating the slot but not the opposite side was drilled into the side of each arm. The hole in the 60-mm. arm was made 26 mm. from the angle, and the one in the handle was made 22 mm. from the angle. The holes were tapped to fit brass screws 3 mm. in diameter. The screws were filed so they were even with the surface of the rod when they were in position. New slots were cut in the outer ends of the screws so they could be tightened with a screw driver. A double-edged razor blade was prepared for use in the holder by bending the blade lengthwise so that it broke longitudinally into two equal pieces. This left a small projection on each end, on the side opposite the cutting edge. These were broken off without removing any of the cutting edge so the blade would fit into the holder, as in figure A. The screws were tightened just enough to touch the blade since too much pressure would splinter the brittle steel.

The blade can be held in the slots by using sealing wax or beeswax but the screws permit a more rapid replacement of

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[1]The author expresses his appreciation to Frank L. Huey, Alabama Polytechnic Institute, for making an earlier model and the enterotome knife described above.

dull blades. Before using, the mesentery must be cut away close to the intestine to allow the gut to be pulled smoothly over the knife. The knife can be used in the hand by inserting the rounded point into the lumen of the gut and pushing it along the intestine. However, if the knife is clamped to the side of a sink (fig. C), it is then possible to use both hands for pulling the intestine upward and thus progress about twice as fast. A regular or swivel clamp fastens the knife handle to the shank of a universal clamp which is attached to the edge of the sink.

A simpler enterotome knife can be used in cutting intestines of smaller animals. This was made by adding a rounded point to a Bard-Parker No. 12 blade (fig. B). The tip of a short length of rosin-cored solder was held in a flame until a rounded knob was formed at the end. The solder was then cut so that the knob and the shank were approximately 14 mm. long. The tip of the blade was then inserted into the open end of the shank and the sides were hammered lightly until the tip was firmly attached. This knife is used in the same manner as the larger enterotome knife.

A Study of Some Effects of Surgical Anesthetic Doses of Delvinal Sodium in the Dog

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COPE AND HANCOCK[1] have prepared and tested, pharmacologically, a number of new substituted vinyl barbituric acids. One of these, 5-ethyl 5-(1-methyl 1-butenyl) barbituric acid (Delvinal)*, has proved to be a valuable sedative and hypnotic, corresponding somewhat to amytal in duration of action. Hendrix,[2] Davidoff,[3] and Davidoff and Goodstone[4] have demonstrated that the new derivative is outstanding because of safeness, lack of exciting, toxic, or after effects and that it has many important advantages in clinical use. The data obtained on dogs by Hendrix[2] proved that it is a good sedative for these animals. Using the loss and recovery of the righting reflex, he determined the a.d. 50 and a.d. 100 in dogs. These values were sufficiently removed from the l.d. 50 and l.d. 100 to indicate that the drug possesses a high therapeutic index. There were no gross or microscopic lesions which could be attributed to the drug even when given three times weekly for eleven weeks; under these conditions, there was no evidence of the development of tolerance. The barbiturate depressed the blood pressure and the respiration in dogs less than amytal. Only negligible amounts could be recovered in the urine, a finding which indicated that Delvinal is almost completely metabolized.

Further information was desired on the effects of Delvinal Sodium in dogs, particularly when given in doses producing surgical anesthesia. This dosage was determined, therefore, and its effects upon the periods of anesthesia and recovery and upon temperature, pulse, and respiration were observed and compared with those produced by the sodium salts of pentothal, pentobarbital, and amytal. The effects of single and of repeated doses upon these variables and upon blood cells, blood glucose, blood and urine, urea and uric acid, and upon the specific gravity of the urine were also studied.

METHODS

Normal male and female dogs weighing from 3.5 to 32.0 kg. were hosptialized and kept under observation for at least two weeks before they were used for experimentation. Each dog was rested for a minimum period of three weeks following each injection of a barbiturate, except when tolerance to the drug was under investigation. Food and water were withheld for eighteen hours prior to the administration of the drug. During anesthesia the dogs were kept in kennels removed from the floor and at a temperature of 25 ± 2 C.

In the preliminary experiments to determine approximately the surgical anesthetic and lethal doses, a solution of Delvinal Sodium (64.8 mg. per cc.) was injected intravenously over a period of not more than twenty minutes until the various reflexes were abolished. In the subsequent experiments, the solutions were injected intravenously within one minute. Observations on anesthetized dogs were made in the following order: respiratory rate per minute, pulse rate per minute, temperature, reflex responses, and body movement.

The hemoglobin, numbers of erythrocytes and leucocytes, and the differentiation of the latter, were determined on blood samples by the technique described by Morris, Allison, and Green.[5] Urea nitrogen, uric acid, and glucose were determined on unlaked, whole-blood filtrates, as recommended by Folin and Svedberg.[6] The permutit method of Wakeman and Morrell[7] was used for the urea nitrogen and the Folin's[8] indirect method for the uric acid determinations in the urine samples.

These studies were made possible by the establishment of a Sharp and Dohme Fellowship in the Department of Physiology and Biochemistry, and are a contribution from the Bureau of Biological Research, Rutgers University.

From the Department of Physiology and Biochemistry, Rutgers University (Allison and Seeley) and the Raritan Hospital for Animals (Morris).

*Delvinal Sodium is Sharp and Dohme's trademark for their brand of the sodium salt of 5-ethyl 5-(1-methyl 1-butenyl) barbituric acid (vinbarbital).

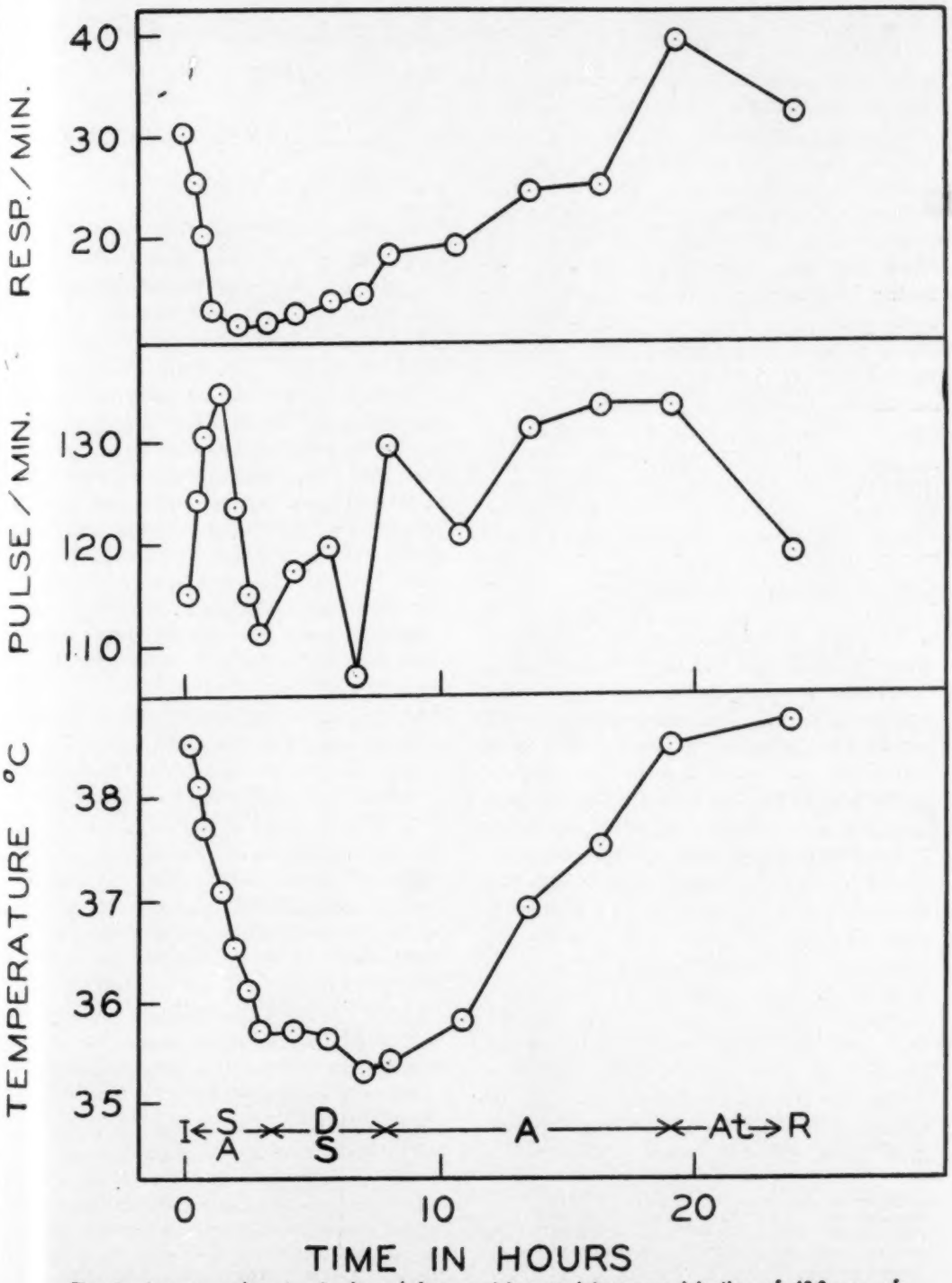

Fig. 1—Average values in six dogs (after receiving an intravenous injection of 48.8 mg. of Delvinal Sodium per kg.) for temperature, pulse rate per minute, and respiratory rate per minute, during the various periods of anesthesia and recovery, plotted against time in hours. The average limits of these periods are marked by the arrows at the bottom of the figure.

I. refers to induction time, S.A. to surgical anesthesia, D.S. to deep sleep, A. to awakening, At. to motor ataxia, and R. to total time.

RESULTS

Dosages.—In the first experiments, the solution of Delvinal Sodium was injected slowly into the vein until the eye reflexes were just abolished. In 10 dogs, an average of 48.8 mg. per kg. of body weight was required to abolish these reflexes. Abdominal surgery was possible, even though the eye reflexes were not entirely lost, when sufficient drug was given to abolish responses to the insertion of a needle into the pads of the paws. The disappearance of the muscular twitches was taken, therefore, as a measure of surgical anesthesia. When 48.8 mg. per kg. were injected intravenously into another group of 20 dogs, 16 of them lost these leg muscle reflexes entirely, whereas, 4 responded slightly to the pain stimulus. This dosage was, therefore, close to the surgical a.d. 100 in these animals. The l.d.o. was determined to be 52 mg. and the l.d. 100 to be 90 mg. per kg. in another group of 18 dogs. Thus 48.8 mg. per kg. was sufficiently removed from the l.d.o. to be used safely for surgical anesthesia.

TABLE 1—Effects in Six Dogs of Intravenous Injections of 48.8 mg. of Delvinal Sodium per kg. of Body Weight upon the Duration of Successive Periods of Anesthesia and Recovery

	Duration of					
	(I) Hours	(S.A.) Hours	(D.S.) Hours	(A) Hours	(At.) Hours	(R) Hours
Minimum	0.25	1.0	0.5	6.0	3.5	13.0
Maximum	1.0	2.5	15.0	16.0	6.0	33.0
Average	0.54	2.3	5.1	11.0	4.9	23.8
Standard Error	0.16	0.91	4.52	3.4	0.55	6.0

(I) = Induction; (S.A.) = Surgical Anesthesia; (D.S.) = Deep Sleep; (A) = Awakening; (At.) = Motor Ataxia; (R) = Total Time.

The intravenous injection of 25 mg. per kg. of the barbiturate produced relaxation and sleep without surgical anesthesia. This dosage is similar to the a.d. 100 for Delvinal Sodium in dogs reported by Hendrix,[2] who used the loss and recovery of the righting reflex to measure the onset and end of anesthesia, respectively.

No gross abnormalities could be found in the dogs, upon which laparotomy was performed during anesthesia, other than the enlargement of the spleen, which is characteristic of barbiturate narcosis. Autopsy of dogs given lethal doses showed no abnormalities, except for the enlargement of the spleen and occasional hemorrhagic areas in the mucosa of the stomach, which were seen to be localized zones of increased vascularity. Microscopic examination of the gastric tissue showed no pathological changes.

ANESTHESIA AND RECOVERY

Table 1 contains a summary of the effects, in 6 dogs varying in weight from 5.4 to 15.7 kg., of the intravenous injection of 48.8 mg. per kg. of body weight upon the duration of successive periods of anesthesia and recovery.

After receiving the barbiturate, the animals relaxed immediately into a state of anesthesia without evidence of excitement. The onset of surgical anesthesia was determined by the disappearance of twitches in the muscles of the legs when a needle was thrust into the paws. Surgical anesthesia lasted until a response to this painful stimulus was again detected. The end of the period of deep sleep which followed surgical anesthesia was determined by the appearance of body movements such as stretching or shivering reflexes, one or the other of these usually appearing first. The first movements of "awakening" often were followed successively by a running motion of the legs while the dog was still unconscious and lying on its side, by attempts to crawl as consciousness was regained, and finally by the establishment of the righting reflex. The total time was measured as the hours elapsing between the injection and the disappearance of motor ataxia which followed the righting reflex.

The effect of Delvinal Sodium upon the duration of the periods of anesthesia and recovery is compared in table 2 with three other barbiturates often used in veterinary medicine. The quantities injected were near the safe maximum dose for each anesthetic. Although the data were secured in but two dogs (6 and 7), the lengths of the periods recorded in this table are representative of those found generally. Thus, although Delvinal Sodium should be classified with amytal and pentobarbital as a hypnotic of moderate duration, a longer period of surgical anesthesia can be obtained from a

single large dose of this new barbiturate than from the others. Peoples and Carmichael[9] also obtained a long period of surgical anesthesia (3 to 5 hr.) in the monkey from a single dose of 40 to 45 mg. per kg.

TABLE 2—A Comparison between the Effects of Four Barbiturates* in Dogs 6 and 7, upon the Times for Induction, Surgical Anesthesia, Deep Sleep, "Awakening," and Motor Ataxia

Sodium Salt of	Dosage	Duration of Period of (I)	(S.A.)	(D.S.)	(A)	(At.)	(R)
	Mg./Kg.	Hr.	Hr.	Hr.	Hr.	Hr.	Hr.
Pentothal	28.6	0.04	0.09	0.70	0.96	0.91	2.7
Pentobarbital	28.6	0.13	0.62	1.95	2.70	2.50	7.9
Amytal	53.5	0.33	0.67	3.50	8.00	5.50	18.0
Delvinal	48.8	0.50	2.50	8.20	13.00	4.00	28.2

(I) = Induction; (S.A.) = Surgical Anesthesia; (D.S.) = Deep Sleep; (A) = Awakening; (At.) = Motor Ataxia; (R) = Total Time.
*The dose of each barbiturate was near that of the maximum safe dose.

injected intraperitoneally. It is interesting to note that even though the periods of anesthesia were longer with Delvinal, the period of ataxia was relatively short in comparison to the others, occupying only about 14 per cent of the total time. The periods of motor ataxia with pentothal, pentobarbital, and amytal are all approximately 30 per cent of the total time. Another outstanding difference is the lack of excitement, that is, lack of annoying reactions or violent movements during the induction and awakening periods following the injection of Delvinal. This barbiturate could be given to the dogs in the hospital, therefore, without preanesthetic treatment with morphine and atropine which are used commonly with other barbiturates to prevent excitement.

The effects of the smaller dosage (25 mg. per kg.), used to produce deep sleep, on the periods of anesthesia and recovery can be illustrated by averages secured in the two dogs (6 and 7). These averages were: deep sleep, 3 hours; "awakening" period, 2.5 hours; motor ataxia, 3 hours; and total time, 8.5 hours. Again, these periods were marked by lack of excitement, an observation which agrees with the reports of others that this new barbiturate is notable for quietness of the sedation and absence of after effects.

Pulse, Respiration, and Temperature

In the study of the periods of anesthesia and recovery, measurements were made of the rectal temperature, pulse, and respiratory rate. The intervals between measurements were: fifteen minutes for the first hour; hourly for the next five hours; two hours for the following four hours; and then every three hours until recovery. The values for these variables at the beginning, quarter distances, and at the end of each period for each dog were obtained from plots and the data averaged. These averages are illustrated in figure 1 where temperature, pulse rate, and respiratory rate per minute are plotted against time in hours. The means of the various periods of anesthesia and recovery are marked by the arrows at the bottom of the figure, where the letters refer to the periods in table 1.

TABLE 3—A Comparison of Minimum Values, Following Injection of Various Barbiturates*

Sodium Salt of	Temperature Minimum for Dog No. (6)	(7)	Pulse Rate Minimum for Dog No. (6)	(7)	Respiratory Rate Minimum for Dog No. (6)	(7)
			per min.	per min.	per min.	per min.
Pentothal	37.2	37.9	90	108	10	20
Pentobarbital	37.5	37.9	84	90	9	20
Amytal	36.8	36.7	84	84	8	10
Delvinal	34.9	34.4	96	120	8	12

*Maximum safe dosages were used.

Curves similar to those in figure 1 were obtained for pentothal, pentobarbital, and amytal. Decrease in temperature, pulse rate, and respiratory rate are common in all barbiturates and are attributed to the quieting action of sleep, lowered metabolism, and after the injection of large doses, to specific affects on nerve centers (Tatum,[10] Goodman and Gilman,[11] and papers quoted therein). A comparison is made in table 3 between the minimum values for temperature, pulse rate, and respiratory rate, following the injection of maximum, safe doses of the four barbiturates, in dogs 6 and 7. These data, which supplement those given for the same dogs in table 2, show that the longer acting barbiturates produce a greater depression in body temperature.

The curves for temperature, pulse rate,

and respiratory rate with the smaller dosage of Delvinal Sodium (25 mg. per kg.) were similar to those shown in figure 1, except that the initial rise in pulse rate following the administration of the barbiturate did not occur and the drop in temperature was not as great, averaging only 1.4 C. below normal in dogs 6 and 7.

To determine the effects of repeated large doses of Delvinal Sodium on the lengths of the periods of anesthesia and recovery, five dogs weighing 13, 9, 22, 9, and 17 kg., respectively, were given weekly injections of 48.8 mg. per kg. These data are illustrated separately for each dog in figure 2. The disappearance of surgical anesthesia after the third week in dogs 1 and 2 and the progressive shortening of the total time for recovery in dogs 2 and 3 may be significant, although all the changes are within normal variation. Since there is no consistent and progressive shortening in all the periods of anesthesia and of recovery in these five animals, it is not possible to interpret those changes which occurred as proof of the development of tolerance to the drug. Furthermore, the effects observed each week on pulse, respiration, and temperature showed no progressive change but remained essentially constant. Similarly, Hendrix[2] found no changes in the effects of Delvinal on dogs, when a smaller dose (21 to 22 mg. per kg.) was administered every other week-day for eleven weeks.

Blood Cytology

During these experiments, the Gm. of hemoglobin, the numbers of erythrocytes, leucocytes, and the differentiation of the latter were determined before, during, and after anesthesia, on the bloods of dogs 1, 2, and 3 receiving weekly injections. The values obtained in the unanesthetized animals did not vary during the weeks the dogs were under observation. The averages, for example, before and after the five

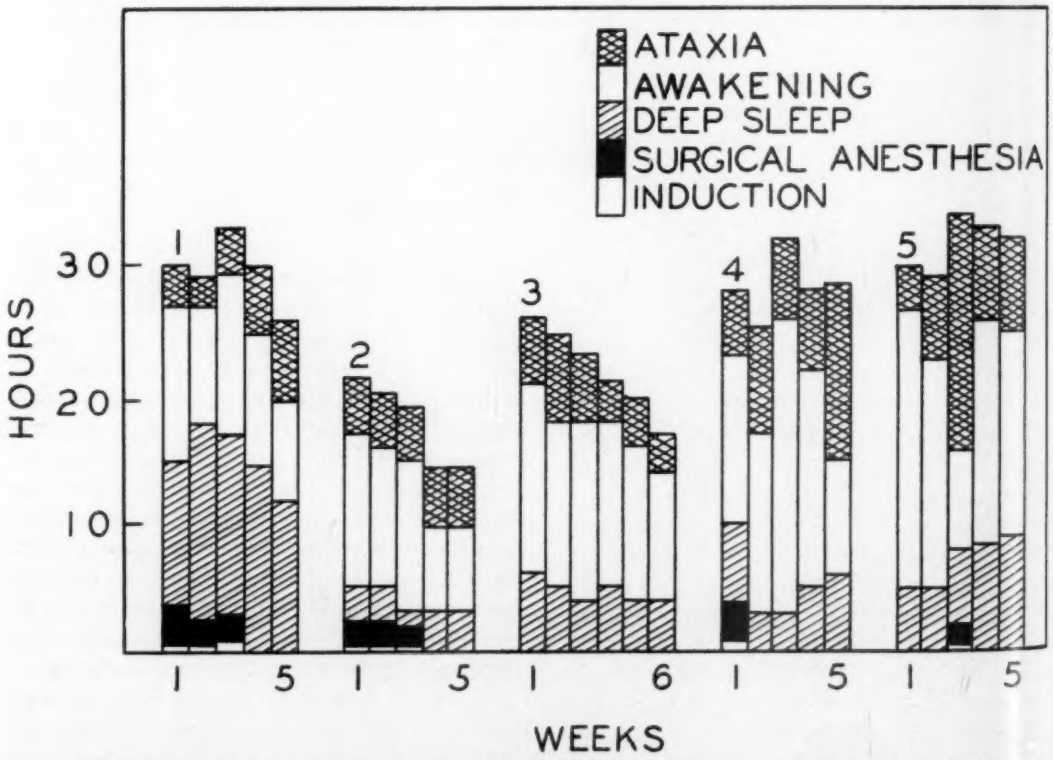

Fig. 2—Duration in hours of the periods of anesthesia and recovery in five dogs (after weekly intravenous injection of 48.8 mg. of Delvinal Sodium per kg.) are charted by weeks. The legend in the upper part of the figure gives the method of indicating the different periods of anesthesia and recovery and the dog numbers are placed above the appropriate group of bars.

weekly injections were 13 and 14 Gm. of hemoglobin per 100 cc., 5.8 and 5.8 millions erythrocytes per cmm., and 18.1 and 18.2 thousands leucocytes per cmm., respectively.

There was a decrease in the number of erythrocytes and of leucocytes during anesthesia each week, the number returning to normal at recovery. These decreases are illustrated by the averages, recorded in table 4, of three weekly determinations made upon the blood of dog 3. Since there was no significant change from normal in the corpuscular hemoglobin, the reduction in hemoglobin was due to the lower numbers of erythrocytes. Similar decreases were found after intravenous injections of a smaller dose, 25 mg. per kg. The average normal number of erythrocytes, for example, in dogs 6 and 7 were 6.7 millions per cmm. Two hours after the injection of the smaller dose, the average had been reduced to 5.83, and upon recovery it was normal again. Similar changes in erythrocytes have been noted in studies on other barbiturates. The work of Bourne, Bruger, and Dreyer[12]; Adolph and Gerbasi[13]; Hausner, Essex, and Mann[14]; Essex, Seeley, Higgins, and Corwin[15]; Bollman, Svirbeley, and Mann[16]; Searles[17]; and others, have shown that the decrease in hemoglobin and erythrocytes during barbiturate anesthesia is due to the temporary removal of these corpuscular elements from the circulating blood by the dilated spleen and to an increase of fluid plasma.

Undoubtedly, the same factors which caused the transient decrease in erythrocytes caused the temporary decrease in leucocytes, which was observed during anesthesia from large doses of the drug. Higgins and Corwin[18] came to the conclusion that there was sequestration of leucocytes in the spleen of the rat, but not in the rabbit, under amytal anesthesia and that the reduction in numbers of white cells which they observed in these animals was due in whole or in part to an increase in plasma volume. The reduction in numbers of leucocytes was not significant, however, during anesthesia from the small dose of Delvinal (25 mg. per kg.); this might be interpreted to mean that there was little or no change in plasma volume at this lower dosage.

TABLE 4—Effects of the Intravenous Injection of 48.8 g. of Delvinal Sodium per kg. upon the Hemoglobin, Numbers of Erythrocytes and Leucocytes (these data are averages of three successive experiments on dog 3)

Hours After Injection	Condition of Dog	Hemoglobin	Erythrocytes	Leucocytes
		Gm. per 100 cc.	Millions per cmm.	Thousands per cmm.
0	Normal	14.6	6.2	15.9
2	Deep sleep	12.3	5.3	9.7
6	Awakening	12.6	5.1	10.7
15	Recovered	14.3	6.3	16.0

The percentage of the various kinds of leucocytes did not deviate from normal during anesthesia in most of the animals. The averages, for example, of one determination on each of three dogs before, during, and after anesthesia were, respectively, segmented forms, 58, 56, and 55 per cent; stab forms, 6, 4, and 7 per cent; lymphocytes, 27, 31, and 32 per cent; eosinophiles, 7, 5, and 3 per cent; and basophiles, 1, 3, and 1 per cent. There was a tendency, however, in two of the dogs after the third weekly dosage for the percentage of lymphocytes to increase from 10 to 20 per cent above normal during anesthesia. More data are needed to determine whether these changes are significant.

Blood Glucose.—The concentration of glucose in the blood of two animals receiving monthly, and three receiving weekly injections of Delvinal was determined at intervals of two hours during the periods of anesthesia and recovery. The data for each dog were plotted and an average curve based on the first two experiments on the five dogs is presented in figure 3. After the injections, the concentration of glucose in the bloods of these dogs fluctuated considerably, with an average increase during the period of surgical anesthesia followed by an average decrease below the initial value during deep sleep. The concentration of glucose in the blood generally increased, although not always, to the original concentration at the time of recovery. Repeated injections did not alter the general picture.

The decrease in blood sugar during the

early recovery periods from surgical anesthesia following the injection of large doses is consistent with observations made by Hrubetz and Blackberg,[19] who studied the influence of barbiturates on blood-sugar

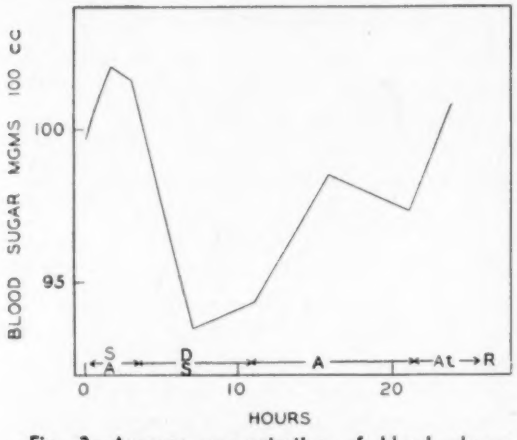

Fig. 3—Average concentration of blood glucose (mg. per 100 cc.) in five dogs (after the intravenous injection of 48.8 mg. of Delvinal Sodium per kg.), during the various periods of anesthesia and recovery, is plotted against time in hours. The average limits of the periods of anesthesia and recovery are marked by the arrows at the bottom of the figure.

concentration and carbohydrate mobilization in the rabbit. They found that some barbiturates had no effect, others increased the concentration of blood sugar during anesthesia, but that all reduced the blood-sugar level at the time of recovery from anesthesia. They demonstrated a relatively small effect of epinephrine on the blood-sugar concentration during barbiturate anesthesia which was interpreted as due to a depression of the glycogenolytic power of the liver. Possibly, the fall in the blood glucose after barbiturate anesthesia was due in part to the utilization of this metabolite in the dogs at a time when the regulatory powers of the liver were not functioning efficiently.

Blood and Urine Urea and Uric Acid

The average concentration of blood-urea nitrogen in dogs 1, 2, and 3 before each of the weekly injections was 17.3 mg., during anesthesia 17.6 mg., and after each recovery period was 16.9 mg. per 100 cc. of blood. The average concentration of blood uric acid before each of the weekly injections in these same dogs was 0.3 mg., and after each recovery period was 0.28 mg. per 100 cc. of blood. Thus, repeated injections did not alter the concentrations of these substances in the blood. Similar results were obtained by Gouaux, Cordill and Eaton[20] with amytal, and by Hefkesbring, Greisheimer and Magalhaes[21] with pentobarbital. The average concentrations of urea nitrogen in the urine of these dogs before and during anesthesia were, respectively, 914 and 1,003 mg. per 100 cc. Under these same conditions, the average concentrations of uric acid were, respectively, 7.0 and 7.26 mg. per 100 cc. of urine. The slight increase in concentration of urea and uric acid in the urine during anesthesia was due, at least in part, to the decreased water content of urine during this period. The average specific gravity, for example, of the urine of these dogs collected during anesthesia was 1.037, which can be compared with an average of 1.029 found for samples collected before and after each experiment. An increase in the specific gravity of the urine during anesthesia could be predicted because of the well-known antidiuretic effects of the barbiturates. The maintenance of normal concentrations of urea and uric acid in the blood and urine, together with the retention of the ability to concentrate the urine during Delvinal anesthesia, are good indications that there was no damage to the kidney or the liver.[22,23]

Summary

1) The intravenous injection of 48.8 mg. of Delvinal Sodium per kg. of body weight produced surgical anesthesia in most dogs while 25 mg. per kg. produced relaxation and sleep. There were no evidences of toxic or after effects.

2) The average duration of successive periods of anesthesia and recovery was determined for both dosages. These averages proved that although Delvinal Sodium should be classed as a hypnotic of moderate duration, a longer period of surgical anesthesia can be obtained in dogs with this barbiturate than with amytal or pentobarbital. This new barbiturate produced no annoying reaction or violent movements

during the induction and recovery periods and could be given, therefore, without preanesthetic treatment with morphine and atropine.

3) The temperature, pulse rate, and respiratory rate were depressed during the periods of anesthesia, rose toward normal during the recovery periods, and were completely normal at the time of recovery. These effects are similar to those produced by other barbiturates.

4) There were no consistent changes in all the stages of anesthesia and recovery in five dogs receiving weekly, surgically anesthetic doses, which could be interpreted as proving the development of tolerance to this barbiturate. The effect on the temperature, pulse rate, and respiratory rate remained unchanged during the five weeks the dogs were under experimentation.

5) A reduction occurred in the hemoglobin content and in the number of erythrocytes and leucocytes during the periods of anesthesia and early recovery; a complete return to normal accompanied full recovery from the drug. These data also failed to indicate that there was a development of tolerance in this species.

6) Following the injection of the large doses of the barbiturate, the concentration of glucose in the blood fluctuated considerably; the averages showed an increase during the period of surgical anesthesia, followed by a decrease below the initial value during deep sleep.

7) The concentration of blood-urea nitrogen and uric acid and the excretion of these substances remained normal, unaffected by weekly doses of Delvinal Sodium. The average specific gravity of the urine increased slightly during anesthesia but always returned to normal, with recovery from anesthesia.

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An Improved Method for Sporulating Oöcysts in Bovine Fecal Material

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IN WORKING with bovine coccidia, it has been found that preparation of fecal material containing oöcysts for experimental infections may be efficiently accomplished by a method which involves screening and aëration. The use of fine sieves to reduce the amount of débris was reported by Goff[1] in work on coccidiosis of chickens, and aëration by bubbling air through the material containing oöcysts to bring about sporulation was done in connection with coccidia of rabbits by Smetana[2] and in work on bovine coccidia by Boughton.[3] The method reported herein consists of the use of a series of fine sieves, followed by a modification of the aëration technique described by Boughton.[3]

In the routine sampling procedure at this laboratory, each fecal sample was washed through a 60 mesh sieve, allowed to sediment, and a portion of the sediment examined by the sugar flotation technique. Samples found to contain significant numbers of oöcysts of the species desired for use in experimental infections were washed through a 100-mesh sieve and a 200-mesh sieve in series, allowed to sediment, then washed through a 325-mesh sieve (openings 44 μ). For each screening of a sample originally containing 100 to 200 cc. of sediment, a total of 2 to 4 liters of water was sprayed through the sieve by means of a rubber tube attached to a faucet. A period of twelve to twenty-four hours was allowed for each sedimentation. The amount of débris eliminated by the screenings varied from 1/4 to 1/2 of the total quantity of sediment, depending upon the coarseness of the particles. Examination of the débris which failed to pass through the screens showed that only very small numbers of oöcysts were lost by screening.

The use of the fine sieves was not essential but resulted in several advantages. In the screened material: (1) Less sedimentation occurred during aëration; (2) there was less difficulty in estimating the number of oöcysts in the material as a result of the absence of the coarser débris; and (3) the oöcysts were relatively more concentrated.

After screening, the sediment was transferred to round-bottomed tubes, each with a capacity of 675 cc., an outside diameter of 5 cm., and a length of 60 cm. An aqueous solution of 2 per cent potassium dichromate was added, and the tubes filled about half way and fastened in an upright position with clamp and ring stand. The aërating equipment consisted of a small aquarium aërating pump connected with rubber tubes, leading into a stone air breaker. An air breaker was placed in each test tube so that it lay at the bottom. With the pump in operation, streams of fine air bubbles kept the material well agitated and aërated. One pump supplied enough pressure to aërate three or four tubes, containing a total of up to 800 cc. of sediment. Each tube was covered with a piece of paper during the period of aëration. Once or twice a day the material which had accumulated on the walls of the tube above the fluid level was scraped off into the fluid with a spatula and the small amount of sediment at the bottom of the tube was stirred up. Sporulation was completed in twenty-four to forty-eight hours for *Eimeria cylindrica* and in forty-eight to seventy-two hours for *Eimeria bovis*, *Eimeria zurnii*, and *Eimeria ellipsoidalis*. These are the sporulation intervals of the oöcysts of each of these species after isolation into a drop of clean tap water

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(Christensen[4]). In practice, an extra twenty-four hours was usually allowed to insure as complete sporulation as possible. The percentage of oöcysts completing sporulation in most samples was above that obtained by methods previously tried, and experimental infections following the administration of this material to calves were more uniform than were infections induced by giving oöcysts sporulated by other methods. The method described above is particularly useful where it is desired to sporulate oöcysts of different species separately.

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Nutrient Requirements of Hemophilus Gallinarum

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IN REVIEWING the literature on the bacterial genus Hemophilus, one is impressed with the large number of conflicting observations on its growth requirements and cultural characteristics. The contradictory statements, relative to *Hemophilus gallinarum*, are especially noticeable.

It has long been recognized that *Hemophilus influenzae* requires for its growth a thermolabile and a thermostable substance as accessory growth factors, both of which are present in blood. Davis[1] showed that the thermolabile substance is also found in fresh plant and animal tissue. Thjotta and Avery[17] named the thermolabile substance V-factor, and indicated that growth of *H. influenzae* in broth containing yeast extract is limited to one or two subcultures because of a deficiency of the thermostable substance, *X-factor*. Lwoff and Lwoff[12] demonstrated V-factor to be identical with the coenzymes.

As pointed out by Thjotta and Avery,[17] potatoes as well as other vegetable tissue contain X-factor. These authors showed that *H. influenzae* could be maintained indefinitely in broth containing small pieces of raw potato.

Although Nelson[14] obtained growth of *H. gallinarum* in blood at the base of nutrient-agar slants, he was not able to cultivate these organisms in broth containing X- and V-factors of plant origin. Delaplane, Erwin, and Stuart[3] were likewise unsuccessful, and Eliot and Lewis[7] found that *H. gallinarum* would not grow satisfactorily in mediums in which the required growth factors of *H. influenzae* were supplied by tissue extracts and hemoglobin. Kessens,[10] working with a European fowl coryza organism which he called *Hemophilus coryzae*, obtained good growth in broth, containing small pieces of raw potato. In a personal communication, Delaplane[2] stated: " . . . the organisms of *Hemophilus gallinarum* do not grow in broth nor colonize under ordinary conditions." Using a raw, potato-broth medium, Gregory[8] obtained excellent growth with several American strains of *H. gallinarum*.

Schalm and Beach,[15] as well as Delaplane, Erwin, and Stuart[4] reported that *H. gallinarum* required both X- and V-factors for growth. In the same paper, Delaplane and associates noted that the presence of salt is essential for growth, and that growth in serum broth was stimulated by the presence of agar. They also obtained growth of *H. gallinarum* in yeast extract at the base of nutrient-agar slants when seeded from blood mediums; however, growth was limited to the first transfer slants unless blood cells or blood extract were added. Prior to the work of these authors, McGaughey[13] isolated hemophilic bacteria from the respiratory tracts of normal fowl and from those affected with coryza. On the basis of their growth in broth containing yeast extract, he assumed they did not require X-factor. Kessens, in the previously mentioned paper, reported that X-factor was not an essential growth factor for his fowl coryza organisms, but that an additional substance which he called the C-factor was essential. Kessens was not able to grow his organisms in broth, fortified with yeast extract and hematin. Delaplane and Stuart[5] were able to grow *H. gallinarum* strains symbiotically or parasitically among growing yeast cells throughout repeated transfers, thus indicating either that yeast produces X-factor

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or that in such a relationship X-factor is not necessary for the continued growth of *H. gallinarum*. Delaplane and Stuart[6] were unable to confirm the existence of a so-called C-factor as being a requirement for their strains.

In view of the above inconsistencies, it was felt advisable to repeat some of the work of others and also to determine the exact nature of the requirements of *H. gallinarum* for accessory growth factors by using more highly purified materials.

GREGORY RESEARCH

TABLE I—Growth of Hemophilus Gallinarum and Hemophilus Influenzae in Yeast Extract—Peptone Water after Twenty-four Hours Incubation

DILUTIONS OF YEAST EXTRACT AND MEDIUM	H. GALLINARUM					H. INFLUENZAE		CONTROL TUBES
	21	31	33	42	44	O	T	
1-25	+	+	+	+	+	3+	3+	o
1-50	*	*	*	*	+	2+	2+	o
1-100	±	±	±	±	*			o
1-500	o	o	o	o	o	o	o	o

o = Growth not visible macroscopally. ± = Questionable growth. * = Very slightly visible growth. + = Light turbidity. 2+ = Slightly heavier turbidity. 3+ = Moderate turbidity.

MATERIALS AND THEIR PREPARATION

As Hoagland and associates[9] pointed out in respect to *H. influenzae*, experimental details are of great importance in the study of growth factors. It is, therefore, deemed advisable to discuss in detail the materials used.

Basal Medium.—To avoid the addition of X-factor through meat extract, 2 per cent Bacto-Peptone* containing 1 per cent NaCl was used. This was tubed in 5 cc. quantities in tubes having a diameter of 5/8 inches and was adjusted to pH 7.3 before sterilization by autoclaving. In preliminary experiments, Proteose-Peptone* and Neopeptone* were compared with Bacto-Peptone but were found to be decidedly inferior for the growth of *H. gallinarum*.

X-Factor.—This factor was added to the basal medium immediately before seeding as chemically pure hemin (Eastman Kodak Co.) dissolved in alkaline, distilled water at the rate of 1 mg. per cc.

V-Factor.—Coenzyme was added to the medium just prior to seeding either as filtered yeast extract or as purified coenzyme I. The yeast extract was prepared by a modified Lwoff technique in which 50 Gm. of "Anheuser-Busch" Brewer's yeast were emulsified in 200 cc. of distilled water and the mixture adjusted to pH 4.5. After storage in the refrigerator overnight, the emulsion was brought to 75 C. in a waterbath and held at 75 to 80 C. for twenty minutes. The yeast cells were thrown to the bottom of the tubes by centrifuging at high speed, and the supernatant fluid filtered through Berkefeld candles and tubed. The extract was adjusted to pH 7.3 immediately before pipetting into the basal medium tubes. Two batches of coenzyme I* were used, each being about 30 per cent pure. The coenzyme was dissolved in M/50 KH_2PO_4 at the rate of 100 mg. per liter and sterilized by passage through Seitz filters. Before being added to the basal medium, the coenzyme solution was adjusted to pH 7.3.

Strains of H. gallinarum.—Strains 21 and 31 were isolated at North Carolina State College, 33 and 42 were obtained from Dr. John Nelson of the Rockefeller Institute, and strain 44 was obtained from Dr. J. P. Delaplane of the Rhode Island State College. All the cultures were carried in this laboratory for varying periods in broth containing small pieces of potato. Seeding of cultures to medium was always accomplished with a 3 mm. platinum loop.

EXPERIMENTAL PROCEDURE AND RESULTS

Growth in Peptone Water Containing Yeast Extract.—In preliminary experiments, it was found that *H. gallinarum* strain 31 grew well throughout repeated transfers in meat-extract broth containing filtered yeast extract (dilution 1 to 25). To determine whether X-factor was present in the medium, two strains of *H. influenzae* (O and T) were secured from Dr. C. L. Hoagland and their cultural reactions in the above medium observed. These strains likewise grew well throughout repeated transfers.

*Manufactured by Difco Laboratories, Detroit, Mich.

As shown in table 1, the *H. gallinarum* and *H. influenzae* strains all grew in yeast extract-Bacto-Peptone water (1 to 25). All strains were subcultured at least 20 consecutive times in this medium, the transfers being made at twenty-four-hour intervals. The growth of the *H. influenzae* strains was considerably heavier than the *H. gallinarum* strains. Growth of *H. gallinarum* strains 21, 31, and 44, as indicated by bacterial swirls when the tubes were lightly tapped, could generally be detected within eight hours. At twenty-four hours, the turbidities produced by these 3 strains were light but always visible. Maximum turbidity usually occurred after twenty-four hours of incubation but before forty-eight hours. The growth of strains 33 and 42 tended to be somewhat variable. Although they sometimes grew just as well as the other strains, they occasionally grew very poorly and the turbidities were negligible. Even when well-defined bacterial swirls of strains 33 and 42 existed after twenty-four hours of incubation, swirls were almost always absent after forty-eight hours. This peculiarity did not exist in the case of the other strains.

Growth of all strains was always good in the first transfers from potato mediums. The growth in the second, third, and sometimes fourth transfers was usually considerably reduced, while in the fifth and subsequent transfers the growth would more nearly approach that obtained in the first transfers.

When the dilution of yeast extract to peptone water was increased to 1 to 50, the growth of all strains was visibly reduced. Except for strain 44, growth of all *H. gallinarum* strains in dilutions of 1 to 100 was quite variable. Strain 44 always produced a slight turbidity, but growth of the others could not always be detected macroscopically. Neither the *H. gallinarum* nor the *H. influenzae* strains produced macroscopically visible growth when the dilution of yeast extract to peptone water was increased to 1 to 500.

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TABLE 2—Growth of Hemophilus Gallinarum and Hemophilus Influenzae in Yeast Extract-Peptone Water Containing 0.01 cc. Hemin Solution per Tube

DILUTIONS OF YEAST EXTRACT AND MEDIUM	H. GALLINARUM					H. INFLUENZAE		CONTROL TUBES
	21	31	33	42	44	O	T	
1-25	+	+	+	+	+	3+	3+	o
1-50	*	*	*	*	+	3+	3+	o
1-100	±	±	±	±	*			o
1-500	o	o	o	o	o	3+	3+	o

o = Growth not visible macroscopally. ± = Questionable growth. * = Very slightly visible growth. + = Light turbidity. 3+ = Moderate turbidity.

Unfortunately (or perhaps fortunately), the Berkefeld filter which had always been used for the filtration of the yeast extract was broken during the latter part of the study. When another candle was substituted, it was found that the extract filtered through it had considerable of its growth promoting properties for *H. influenzae* removed. When diluted 1 to 25 with peptone water, the visible macroscopic growth of strain T was limited to no more than 4 or 5 transfers, and the turbidity produced was always very light. Strain O grew slightly better, and it appeared that growth of this strain would continue indefinitely in this medium. Yeast extract, filtered through Seitz filters, supported the growth of strain O but not T. Substitution of filters apparently had little if any effect on the growth of *H. gallinarum*.

Effects of X-Factor on Growth of H. gallinarum.—There was no clear-cut evidence that the addition of 0.01 cc. of hemin solution to yeast extract-peptone water had any effect on the growth of *H. gallinarum* (table 2). At times, tubes containing added X-fac-

*Coenzyme samples obtained from Dr. Philip Handler, Duke University, Durham, N. C., and Dr. Charles L. Hoagland, Hospital of the Rockefeller Institute for Medical Research, New York, N. Y.

tor appeared to have a slightly heavier turbidity and to develop a turbidity slightly earlier than similar tubes which did not contain added X-factor. However, the reverse was true in probably just as many instances.

The addition of hemin to tubes seeded with *H. influenzae* strains had a noticeable effect, especially in the higher dilutions. With yeast extract filtered through the first-used filter and diluted 1 to 25 with peptone water, the effect of adding X-factor was negligible. However, when added to yeast extract-peptone water, diluted 1 to 500, the turbidity produced was equal to that produced when the dilution was 1 to 25, without added X-factor. With yeast extract filtered through the second Berkefeld candle, addition of X-factor was always effective.

Effect of Percentage of NaCl in Basal Medium.—The effect of various concentrations of NaCl in yeast extract-peptone water on the growth of *H. gallinarum* strains was compared. In this experiment, the inoculum was taken from 24-hour cultures in 10 per cent chicken-blood broth and in broth containing small pieces of potato. As indicated in table 3, the medium containing 1 per cent salt was superior to that containing higher or lower percentages. Growth did not occur in tubes which did not contain salt or in tubes containing 2.5 per cent and 3.0 per cent, respectively. Growth in 2 per cent NaCl medium was limited to two strains. Although the growth in the two types of inoculum appeared to be about equal, tubes seeded from the blood medium always appeared to yield heavier growth except in the case of strain 44. This appears to indicate the heavy concentration in blood cells of an additional growth factor.

The Effect of Agar on Growth in Yeast Extract-Peptone Water.—Since the Delaplane group[4] indicated that agar had a stimulating effect on the growth of *H. gallinarum*, nutrient agar in 15 cc. quantities was poured into Petri dishes and the hardened agar cut into strips of various lengths and widths. These strips were added to tubes of yeast extract-peptone water before seeding with *H. gallinarum*. None of the tests indicated that agar strips enhanced the multiplication of *H. gallinarum*.

Addition of Coenzyme I to the Basal Medium with or without Added Hemin.—Coenzyme was allotted to tubes of basal medium so as to have final concentrations of coenzyme of 2 μg. and 10 μg. per cc., respectively. The inoculum consisted of 18- to 24-hour yeast extract-peptone water cultures. *H. gallinarum* strains 31, 42, and 44 often produced a very faint turbidity after twenty-four to forty-eight hours of incubation in the first transfer tubes. Growth could seldom be detected in subsequent transfers. Strains 21 and 33 never gave evidence of growth in this medium.

The value of adding X-factor to this medium was highly questionable. Growth in first transfer tubes of strains 42 and 44

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TABLE 3—Twenty-four-hour Growth of Hemophilus Gallinarum Strains in Yeast Extract-Peptone Water. (1 to 25) Containing Various Percentages of NaCl

NaCl %	S. 21		S. 31		S. 33		S. 42		S. 44	
	B	P	B	P	B	P	B	P	B	P
0.0%	o	o	o	o	o	o	o	o	o	o
0.5%	+	o	2+	o	+	o	*	o	2+	2+
1.0%	2+	+	2+	+	2+	+	+	+	2+	2+
1.5%	+	*	2+	o	2+	o	*	*	+	+
2.0%	o	o	o	o	+	o	o	o	+	*
2.5%	o	o	o	o	o	o	o	o	o	o
3.0%	o	o	o	o	o	o	o	o	o	o

S = Strain. B = Seeded from 10% blood broth. P = Seeded from broth containing small pieces of raw potato.
o = Growth not visible macroscopally. * = Very slightly visible growth. + = Light turbidity. 2+ = Slightly heavier turbidity.

often seemed to show the effects of slight stimulation, but added X-factor apparently had no effect on the growth of strains 21, 31, and 33.

The V-factor used in these experiments was sufficiently potent as shown by good growth of *H. influenzae* in peptone water containing X-factor and a concentration of 0.02 μg. of coenzyme per cc.

DISCUSSION

Because of all the literature to the contrary, it is highly interesting and surprising to find that both *H. influenzae* and *H. gallinarum* strains grew throughout repeated transfers in peptone water containing a small amount of yeast extract. These studies clearly indicated that material which can function as X-factor in the growth of *H. influenzae* may be present in yeast, and that such material may pass through bacterial filters in sufficient quantity to allow for the growth of *H. influenzae*. Whether all strains or species of yeasts produce this material is not known, but it was most certainly present in the yeast used in these studies. It is generally known that X-factor is adsorbed by bacterial filters during the process of filtration, and these studies proved that certain filters may remove considerable more X-factor from yeast extract than other filters. Perhaps the explanation of the differences in the observations of Thjotta and Avery[16] and of the writer may lie in the method of preparation of yeast extract, in the species of yeast used, or in dissimilar quantitative requirements for X-factor by *H. influenzae* strains.

Apparently, only three groups of workers have previously attempted to grow fowl-coryza organisms in yeast preparations. The failure of Kessens[10] to obtain growth of his *H. coryzae* in broth containing yeast extract can be ascribed to the method of yeast extract preparation. Kessens boiled his extract for ten minutes at pH 7.6, a procedure which certainly would be expected to destroy a great deal of the coenzyme present. The bacteriological technique used by Delaplane and coworkers[4] yielded only limited growth in the first transfer tubes from blood mediums. Several of McGaughey's[13] strains of hemophilic organisms, isolated from chickens with coryza, may have been *H. gallinarum* strains, as they grew well in broth containing yeast extract.

The contention of Delaplane and associates[4] that the presence of salt is necessary for the growth of *H. gallinarum* is further augmented by these studies. Although the above workers found the optimum concentration of NaCl in serum broth to be 1.5 to 2.0 per cent, the writer found a concentration of approximately 1.0 per cent to be the most desirable in the case of yeast extract-peptone water. In fact, 2 per cent salt was found to be highly toxic. These discrepancies can probably be best explained on the basis that Delaplane and associates used their broth at the base of nutrient agar slants which did not contain salt. Such a condition undoubtedly allowed for the diffusion of some NaCl into the agar, and thus the concentrations of salt used were more apparent than real. In addition, the growth obtained by these workers was very poor and necessitated microscopic examination of the cultures, whereas the growth obtained in the present study could easily be detected with the naked eye.

The findings of Delaplane and coworkers,[4] relative to the necessity of the presence of agar for the growth of *H. gallinarum*, can probably be reconciled with the present study only on the basis of differences of employed technique. The writer was not able to detect any property in agar which was stimulating to the growth of *H. gallinarum*.

Neither the need of coenzyme nor of hemin for the growth of *H. gallinarum* has been proved in these experiments, nor in any other experiments of which the writer is cognizant. These studies indicated that yeast extract, when mixed in the proper proportion with peptone water, will support the growth of *H. gallinarum*. Although the yeast extract used in this study clearly contained X- and V-factors, as evidenced by the growth of *H. influenzae*, *H. gallinarum* would not continue to grow in peptone water containing many times over the amount of

purified X- and V-factors required for good growth of *H. influenzae*. The probable need of coenzyme by *H. gallinarum* is indicated, but until such a time when it may be shown that these organisms will or will not grow in relatively purified mediums, containing all the required growth promoting factors other than coenzyme, the statement that V-factor is a required accessory factor for *H. gallinarum* must be held in abeyance.

These studies tend to upset the belief that X-factor is required for the growth of *H. gallinarum*. However, in the event that it is required, the writer feels the present studies indicate X-factor to be necessary only in very minute amounts, even more minute than is required by many *H. influenzae* strains.

The need of other growth factors by *H. gallinarum* was clearly indicated by their failure to give continued growth in peptone water containing large quantities of X- and V-factors. Growth in first transfers from yeast extract preparations suggested a carry-over of some growth promoting material in the yeast extract. In the "salt" experiment conducted by the writer, the fact that *H. gallinarum* generally grew much better when seeded from blood mediums than when seeded from potato mediums tended to show that growth promoting substances, other than X- and V-factors, were present in blood cells in greater concentration than in broth containing a small piece of potato.

Although the need of other accessory factors for the growth of *H. gallinarum* is predicated by this study, the writer does not feel that the C-factor of Kessens[10] is involved. The writer has boiled filtered yeast extract for ten minutes with no apparent impairment of growth promoting qualities for *H. gallinarum*. Although Kessens was working with hemophilic organisms which caused coryza in chickens, it seems unlikely that *H. gallinarum* and *H. coryzae* are identical.

SUMMARY AND CONCLUSIONS

1) A study was made of the nutrient requirements of 5 strains of *Hemophilus gallinarum*.

2) All 5 strains were subcultured at least 20 consecutive times in Bacto-Peptone water containing yeast extract. A light turbidity was produced in this medium which could readily be detected within twenty-four hours of incubation.

3) Two strains of *Hemophilus influenzae* also grew throughout repeated transfers in the yeast extract-peptone water medium, indicating that the yeast extract used contained X-factor.

4) The quantity of X-factor in the filtered yeast extract depended upon the bacterial filter used.

5) The optimum concentration of NaCl for good growth of *H. gallinarum* was found to be about 1 per cent.

6) Agar had no stimulating effect on the growth of *H. gallinarum*.

7) Satisfactory growth of *H. gallinarum* was not obtained in peptone water containing large quantities of purified X- and V-factor.

8) X-factor did not appear to be necessary for the growth of *H. gallinarum* strains used in this study.

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Effects of Environment Upon the Free-Living Stages of Ostertagia Circumcincta (Stadelmann) Trichostrongylidae: 1. Laboratory Experiments

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THE BROWN stomach worm of sheep, *Ostertagia circumcincta* (Stadelmann), is the cause of a great annual loss to the sheep industry in California.[1] The frequent occurrence of acute ostertagiasis in lambs on irrigated pastures, together with the marked seasonal incidence of this helminth in sheep,[2] indicates a need for more exact information concerning the ecology of the free-living stages of the parasite.

Although the literature on the bionomics of trichostrongyles is extensive, only occasional observations on the bionomics of the free-living stages of *O. circumcincta* have been published.

Morgan[3] reported that mature larvae are killed within one-half hour at 45 C. and are quite sluggish at 18 C.

Griffiths[4] stated that this species can withstand winter temperatures ranging from –15 F. to 52.5 F. for at least a four-month period. Apparently, however, these temperatures were not recorded at the soil surface and no account was taken of the insulation afforded by snow, nor was there any definite determination of the stages of the parasite that resisted these temperatures.

The work of Swales[5] confirms the findings of Griffiths that *O. circumcincta* may survive the winter season in eastern Canada.

Tetley,[6] working with *O. circumcincta*, Trichostrongylus spp., and *Haemonchus contortus* in New Zealand, found Ostertagia infection greatest in the season of spring rainfall and the summer following, indicating that peaks of infection in the sheep were correlated with availability of larvae. Rogers[7] stated that infective larvae of Ostertagia migrate better on wet vegetation than do those of *H. contortus*, and in relatively dry, hot weather, Haemonchus larvae are more active and migrate better. This is in accordanee with the findings of Tetley,[6] who showed that infection with *H. contortus* takes place under drier conditions than with *O. circumcincta*.

The following experiments were conducted in an effort to clarify the relationship between degree of incidence of *O. circumcincta* in sheep and environmental factors affecting the free-living stages of the parasite.

METHODS

Parasite-free lambs were reared on bottled cow's milk from one day after birth to the age of 2½ months. The diet was gradually changed from milk to ground barley and alfalfa hay obtained from ground not used for pasturing livestock. The animals were isolated in a pen provided with concrete flooring which was swept every three days. Once a week, the pen was thoroughly scrubbed with a cresol disinfectant to diminish the possibility of accidentally establishing any parasites.

Frequent fecal examinations were conducted to detect any accidental parasitism. The procedure used was a modification of the Stoll flotation method described by Freeborn and Stewart.[1]

The original supply of *O. circumcincta* was obtained from the abomasum of a heavily infected animal. Of the male worms taken from this animal, 98 per cent were *O. circumcincta* and 2 per cent *Ostertagia trifurcata* Ransom. Since the female worms are morphologically inseparable, the larvae cultured from them did not constitute a pure infection. However, for

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practical purposes, larvae obtained from subsequent cultures may be considered to be of one species, *O. circumcincta*, since the small percentage of *O. trifurcata* present would not significantly alter the results of the experiments.

Female worms thus obtained were retained in warm physiological saline in Petri dishes. Twenty-four hours later, the eggs which they had deposited were drawn off with a micropipette and placed in a 2 mm.-deep mixture of boiled sheep feces and water. The cultures were then placed at room temperature (60 to 80 F.) for six to seven days, after which time the infective forms were drawn off in Baermann funnels.

TABLE 1—Constant Temperature and Humidity Chambers Employed

TEMPERATURE (C.)	RELATIVE HUMIDITY (%)	SATURATION DEFICIENCY (MM. HG.)	SOLUTION
45	72	20.13	Saturated NH_4Cl
37	85.3	7.11	Glycerine 46.7 per cent by wt.
37	59	19.8	Glycerine 79.0 per cent by wt.
27	73.4	7.11	Saturated NaCl
27	26	19 8	Saturated $CaCl_2$
5	77	1.5	Saturated NaCl
—6	80	0.586	Saturated NaCl

Parasite-free lambs 1 to 2 months of age were infected with approximately 10,000 infective larvae obtained from the above-mentioned cultures. After twenty to thirty days, feces were collected from the animals and cultured to obtain infective larvae.

At the termination of the experiments, autopsies were performed on the infected lambs and the entire gastrointestinal tract examined. With one exception, the results of these examinations were negative for species other than Ostertagia. In the last of the experimental animals, 10 adult *Trichostrongylus axei* were recovered. Since this infection was light and occurred only in the last of the experimental animals, it is improbable that it appreciably altered the results of the experiments.

Laboratory tests on the free-living stages of *O. circumcincta* were conducted in constant temperature and humidity chambers. Various solutions (table 1) were used to control the saturation deficiency of the air in the containers. The humidity chambers used varied in size from a volume of 0.5625 to 0.6512 cu. ft. Each chamber was a closed unit with no mechanical agitation of the contained air. The humidity was frequently checked by means of corrected dial hygrometers. The readings were maintained accurate to + or – 3 per cent relative humidity. The temperature chambers remained constant within + or – 2 C. Mechanical agitation of the air was provided by electric fans.

It has seemed advisable to employ the term saturation deficiency expressed in mm. of mercury rather than per cent of relative humidity, in order best to compare the relative evaporating powers of air at different temperatures.

In table 1, both relative humidity and saturation deficiency readings are given for the sake of clarity.

Detailed descriptions of experimental technique are given below, with the appropriate sections to which they pertain.

EFFECTS OF TEMPERATURE AND MOISTURE ON NONEMBRYONATED AND EMBRYONATED EGGS

Viable eggs of *O. circumcincta* were obtained from the infected lambs by utilizing a modification of a method given by Faust *et al.*[8] Fresh sheep feces were mixed with water until of a fluid consistency. This mixture was sieved through a screen having forty meshes to the linear inch. The sieved feces were diluted and centrifuged at approximately 2000 r.p.m. two to four times, retaining the sediment. To the remaining sediment was added calcium chloride solution (specific gravity 1.228) in sufficient quantity to fill the tube. The material was mixed, centrifuged, and the eggs removed from the surface with a wire loop. The eggs were stored in shallow water at room temperature until used.

Laboratory tests on the effects of temperature and humidity on nonembryonated and embryonated eggs were conducted with 100 eggs per test. The eggs were placed on slides by means of a micropipette. Those to be used in testing the effects of saturation deficiency were dried in an air stream until there was a barely visible water film remaining.* The eggs were then immediately placed under the experimental conditions. Eggs to be tested in water at various temperatures were retained on slides in a drop of water. At stated intervals, slides were removed from the experimental environments, distilled water was added, and any further development at room temperature (60 to 80 F.) noted. Eggs not developing or hatching within forty-eight hours

*Experiments conducted by the Zoölogical Division, Beltsville Research Center, Beltsville, Md., indicate that the drying of eggs, by itself, is an important factor in determining the hatchability of the eggs.—Ed.

after removal to room temperature were considered dead.

The results of these tests are given in tables 2 and 3. It is apparent that, in general, temperature extremes and desiccation affect both nonembryonated eggs and embryonated eggs to approximately the same extent. However, embryonated eggs in water demonstrated a much greater resistance to temperatures just above freezing than did nonembryonated eggs.

Larvae to be used in testing the effects of saturation deficiency were placed on microscope slides at the rate of 50 larvae per slide. Further treatment was the same as that just described for eggs under similar circumstances. Viability was determined by observing movement of the larvae after removal from the experimental conditions and soaking for from one hour to two days in water at room temperature (60 to 80 F.). It was noted that a slapping motion imparted by *minuten nadeln* affixed to wooden handles induced motion in some larvae which otherwise were motionless.

TABLE 2—Effects of Temperature and Moisture on Nonembryonated Eggs

		Temperature											
		—6 C.		5 C.		27 C.			37 C.			45 C.	
		Saturation Deficiency (in mm. mercury)											
		Ice	0.586	H_2O	1.5	H_2O	7.11	19.8	H_2O	7.11	19.8	H_2O	20.13
Exposure		Per cent Mortality											
Hours	Days												
1					55		62	98		92	100	18	100
2			60		55								
4					55		86	100		100		54	
6							98					96	
	1	80	60		62		100		44			100	
	2					22			62				
	3		93										
	6		95		70								
	7	90		22									
	10	93											
	11		100		88								
	14	93											
	16				100								
	22	100											
	24			94									
	32			96									
	63			100									

Effects of Temperature and Moisture on Preinfective and Infective Larvae

Eggs obtained by the calcium chloride flotation technique previously described were cultured in a 5 mm.-deep decoction prepared from sheep feces. From these cultures, preinfective larvae were isolated for the following laboratory tests within six hours from the time of hatching. Those to be tested in water, with the exception of larvae to be placed at a temperature of –6 C., were placed in Stender dishes at the rate of 100 larvae per dish. A broth of boiled sheep feces was added to a depth of 0.5 cm. and the dish covers were sealed with vaseline. Larvae to be tested at –6 C. in water (ice) were placed in a series of shell vials at the rate of 20 larvae per vial, in water 0.5 cm. deep. In checking these vials at stated intervals, the first recorded 100 per cent mortality was verified by examination of all remaining vials.

Tests on infective larvae were carried out in the manner described for preinfective forms. The larvae were obtained from fecal cultures under fifteen days of age. Distilled water was used rather than the fecal decoction mentioned previously. Final tests for viability after given intervals were made after soaking the infective larvae in water at room temperature (60 to 80 F.). No larva was discarded in less than forty-eight hours, within which time motion could be detected in any living specimens. Whether or not such larvae would prove infective to sheep was not determined, but for the purposes of this work they were considered alive.

The results of the laboratory tests on preinfective and infective larvae are given in tables 4 and 5 respectively.

From the data given in table 4, it is evident that preinfective larvae are rapidly killed by moderately high temperatures and by very small saturation deficiencies.

A comparison of the results given in table 5 with those of tables 2 to 4 shows that, with one exception, infective larvae were more resistant to saturation deficiency and temperature extremes than any of the preinfective stages. At a temperature of −6 C., infective larvae frozen in ice displayed little or no greater longevity than did nonembryonated or embryonated eggs. This seemed to be due in part to the fact that freezing water mechanically ruptured many of the infective as well as preinfective stages of *O. circumcincta*. This viewpoint is well supported by the fact that infective larvae held at a temperature of −6 C. survived a relatively long time when they were not exposed to freezing water.

TABLE 3—Effects of Temperature and Moisture on Embryonated Eggs

		Temperature											
		−6 C.		5 C.		27 C.			37 C.			45 C.	
		Saturation Deficiency (in mm. mercury)											
		Ice	0.586	H_2O	1.5	H_2O	7.11	19.8	H_2O	7.11	19.8	H_2O	20.13
Exposure Hours	Days	Per cent Mortality											
1												34	100
2			28		10		90	96		94	100		
4			26		30		98			100		80	
8												68	
	1	10	34	10	24	12	100	100	12			94	
	2								6			100	
	3	38				18			4*				
	7	48	96	20	86								
	10	92											
	14	100	100		100								
	22			6									
	32			4									

*Eggs hatched: one to three days.

Tropisms of Infective Larvae

At temperatures of 5, 23, and 37 C., ten-day-old fecal cultures in quart jars were exposed to desiccation by concentrated H_2SO_4. The rapid rate of drying prevented any further migration of colonies present on the jar walls. Identical cultures exposed to conditions of room temperature (60 to 80 F.) and a saturation deficiency ranging from 3.73 to 14.9 mm. of mercury clumped in masses on the sides of the culture jars or migrated downward. The deciding factor in the latter experiments apparently was the rate of drying, since there

TABLE 4—Effects of Temperature and Moisture on Preinfective Larvae

		Temperature									
		−6 C.		5 C.		27 C.		37 C.		45 C.	
		Saturation Deficiency (in mm. mercury)									
		Ice	0.586	H_2O	1.5	H_2O	7.11	H_2O	7.11	H_2O	20.13
Exposure Hours	Days	Per cent Mortality									
1			100		100		100		100		100
	1	100		0		0		0		100	
	2			0		0		50			
	3			0		0		90			
	8			0		0					
	10			51							
	14			57							
	22			68							
	29			80							
	35			91							
	46			100							

was greater migration at a low rather than at a high saturation deficiency.

To determine the existence of geotropism and phototropism of infective larvae, test tubes 6 in. in height by 1 in. in diameter were filled with sterile, moist sand to a depth of 1 in. Two to four thousand larvae were placed on the sand and the tubes were corked. At each temperature under consideration, some tubes were maintained in complete darkness and others supplied with a dim light from a 75-watt electric bulb; the bulb was 5 ft. distant on a parallel plane and was shaded by a single thickness of paper toweling.

The results of these experiments indicated a definite negative geotropism, since larvae kept at a temperature of 23 C. migrated vertically, in either the presence or absence of light, to approximately the same height. The influence of light was seen, however, in that larvae migrating in darkness were uniformly scattered, while those migrating in the presence of a dim light demonstrated a concentration toward the areas of greatest illumination. These results are not wholly in accord with those of Rogers,[7] who reports that there is little or no vertical migration in the dark. No vertical migration of larvae was observed at temperatures of 37 C. and 5 C.

It is evident that in clear, relatively dry weather, the moisture requirements for migration would be best met at the time of dew formation, at night or in the early morning; however, this would be inhibited by a temperature drop nearing the freezing point.

Phototropism of infective larvae on a parallel plane was checked by a method similar to that described by Mönnig[9] and Stewart and Douglas.[10] A glass tube 22 cm. long and 2.5 cm. in diameter was filled with tap water. In this tube were placed several thousand infective larvae. The tube was plugged at both ends and one-half of it was inserted into a black cardboard box. The other end rested on a white paper background and was illuminated from a distance of 25 cm. by a 75-watt electric bulb with a white reflector. The test was repeated, using a tube 7 mm. in diameter and 22 cm. in length.

These experiments demonstrated that there is no phototropic activity of infective larvae when in water in a glass tube.

TABLE 5—Effects of Temperature and Moisture on Longevity of Infective Larvae

Exposure		Temperature											
		—6 C.		5 C.		27 C.			37 C.		45 C.		
		Saturation Deficiency (in mm. mercury)											
		Ice	0.586	H_2O	1.5	H_2O	7.11	19.8	H_2O	7.11	19.8	H_2O	20.13
Hours	Days	Per cent Mortality											
1													100
24				0				6		26	73	0	
	2	0	42	1			1	36	0	81	100	40	
	3						1		0			96	
	5						2					99	
	7	85		1		0	2					100	
	10								12				
	12		71		0.5		16	21		100			
	13								72				
	14	95		1		1							
	15								94				
	20						86		97				
	22	100		1		1		83					
	24								100				
	28		68	1		1							
	40		74										
	42			1		2							
	43						97	98					
	50				15								
	57			1		12							
	70			8		12	100						
	84		87	8	32	34							
	105			9		86							
	135			9		99							
	147			16		100							
	271			17									

VERTICAL MIGRATION AND SURVIVAL OF INFECTIVE LARVAE DEVELOPING IN FECES BURIED IN DIFFERENT SOILS

In order to determine the effects of burial in soil upon the development and migration of the free-living stages of *O. circumcincta*, metal cans 3 to 5 in. in diameter and 6 to 12 in. in height were used. A thin layer of plaster of Paris was applied to the inner walls of the cans. While this was still moist, soil of the type to be tested was imbedded on the walls. This technique was designed to eliminate the possibility of larvae migrating up the sides of the metal containers in condensed moisture during the course of experimentation. The cans were fitted at the top with a paper sleeve. Since the final level of the soil extended above the rim of the can, the upper layers of soil could be sampled easily after removing the paper sleeve. In filling the cans, soil was first added to a level approximately ½ in. below the depth at which the feces were to be buried. Weighed feces of known egg content were then added and buried to the desired depth. The soil used had previously been sterilized by dry heat and approximately 20 per cent by weight of distilled water had been added. Three soil types were used: Yolo sand, Yolo fine sandy loam, and Yolo clay loam. The soils were classified according to the index given by Storie (1937).[11]

The containers were held in a chamber at room temperature (60 to 80 F.) with a saturation deficiency at all times less than 5 mm. of mercury. At weekly intervals, the containers were weighed to determine the amount of water lost through evaporation; by means of a small syringe, water was slowly replaced. A control culture was made of the same lot of feces from which the buried samples were taken. The surface soils were removed to a depth of ¼ in. at stated intervals (table 6), and larvae recovered with the Baermann apparatus. Samples so removed were replaced by sterile moistened soil.

TABLE 6—Relation Between Number of Larvae Recovered from Soil Cans and Number of Eggs Originally Buried

Soil type	Eggs buried (No.)	Distance from surface of feces to surface of soil (In.)	Interval from burial of feces to final examination of surface soil (Days)	A Total larvae recovered from surface soil		Interval from burial of feces to removal and examination of feces and soil from containers (Days)	B Total larvae recovered from feces and soil upon removal from containers		AB Total larvae recovered	Proportion of larvae recovered to eggs buried (approx.)
				No.	%*		No.	%*	No.	%
Yolo Sand	30,000	3	10	28,666	95.56	..	.....	.....	.....	.....
	30,000	9	10	21,600	72.00	74	7,890	26.3	29,490	98.30
	60,000	12	24	19,500	32.50	42	39,625	66.04	59,125	98.54
Yolo Fine Sandy Loam	30,000	3	10	3,100	10.33	..	.....	.....	.....	.....
	30,000	9	27	21†	0.07†	74	24,376	81.25	24,397	81.32
	60,000	12	24	14,932‡	24.89‡	42	30,088	50.14	45,020	75.03
Yolo Clay Loam	30,000	3	10	2,790	9.30	..	.....	.....	.....	.....
	30,000	9	27	3†	0.01†	74	7,223	24.08	7,226	24.09
	60,000	12	24	4,160‡	6.93‡	42	6,052	10.08	10,212	17.02

*Approximate per cent. †Soil screened through ¼-in. mesh and firmly packed. ‡Soil coarsely broken and loosely packed.

The total number of living larvae present in samples buried at 9 to 12 in. in all soil types was determined at the expiration of the experiments for vertical migration (table 6, column B). The soil was removed from the container, thoroughly mixed, the total volume determined, and a 100-cc. sample checked for larvae by means of the Baermann apparatus.

The data on recovery of infective larvae from surface soils and from feces and soil buried for stated intervals of time (table 6) show that eggs of *O. circumcincta* contained in sheep feces buried 3, 9, and 12 in.

deep in Yolo sand, Yolo fine sandy loam, and Yolo clay loam developed to the infective larval stage.

In all soils tested, the length of time required for larvae to reach the surface increased with depth of burial. Of more importance, however, was the rôle played by soil type. As is seen in table 6, column A, sand proved most favorable for larval migration to the surface. The total numbers of larvae recovered from the surface soil of the 12-in. burials (table 6, column A) within twenty-four days show that infective larvae migrate best through sand (32.5%), next through fine sandy loam (24.89%), and least through clay loam (6.93%). Few larvae reached the soil surface after twenty-four days from the time of burial.

The relation between the number of eggs buried and total number of infective larvae recovered is shown in table 6 (column AB). From these data, it is evident that burial in light soils provides a favorable medium for larval development; increasing heaviness of the soil is associated with an increased mortality of preinfective stages buried therein.

During the course of the experiments, it was ascertained that infective larvae were unable to migrate through relatively dry soil.

Since, as is shown in table 6, a large percentage of buried larvae can survive at least seventy-four days in moist soil, it is apparent that burial of larvae during a dry season would favor rather than diminish the longevity of infective larvae due to the protection offered from desiccation in the surface soil. This is in agreement with Taylor's[12] statement that plowing favors the survival of larvae of sheep nematodes.

MIGRATION OF INFECTIVE LARVAE DOWNWARD FROM THE SURFACE OF SOIL

Several thousand larvae were placed on the surface of moist sand and subjected to a saturation deficiency of less than 5 mm. of mercury at room temperature (60 to 70 F.). Examination of soil samples on the seventh day after infestation showed that only a limited number of larvae had migrated downward and almost no larvae had reached a depth greater than 2 in.

Downward migration of infective larvae in soil probably serves as a protective mechanism, since the insulating properties of the soil modify the extremes of heat and the rate of drying to which buried larvae are subjected. This partially explains how infective larvae can survive relatively long periods of unfavorable weather in the summer and reappear on vegetation during the more propitious weather of the fall months.

LATERAL MIGRATION OF INFECTIVE LARVAE ON SOILS OF DIFFERENT TYPES

In order to determine the extent of lateral migration on soils of different types, a Yolo fine sandy loam and a Yolo clay loam were used.

The soils were screened and sterilized with dry heat, following which 20 per cent moisture by weight was added. Two wooden boxes, each 1 ft. square and 5 in. deep, were filled with prepared soil to a depth of approximately 3 in. Approximately 60,000 active, infective larvae were placed in a small depression at the center of the surface soil. At stated intervals, samples of the surface ⅛ in. of soil were examined at a radial distance of 1 to 2 in., 3 to 4 in., and 5 to 5½ in., by means of Baermann funnels. Samples removed were replaced by sterile, moist soil. To obtain the samples, metal hoops with diameters of 1, 2, 3, 4, 5, and 5½ in. were placed concentrically in the soil to a depth of ¼ in., and all the soil within the designated areas removed to a depth of ⅛ in. Throughout the experiment, the soil boxes were maintained in a chamber of low saturation deficiency (5 mm. of mercury or less), and any water loss, determined by weighing the boxes, was replaced by a fine spray applied with an atomizer.

Samples of soil taken at intervals from fourteen to ninety days after infestation showed that practically all migration was within a radius of 1 to 2 in. from the source of infestation. Over three times as many larvae were recovered at a distance of 1 to 2 in. in fine sandy loam as in clay

loam, indicating that infective larvae are able to migrate best through coarse soil.

SUMMARY AND CONCLUSIONS

Nonembryonated and embryonated eggs of *Ostertagia circumcincta* in shallow water (5 mm.) are killed within one to two days by a temperature of 45 C. In ice at –6 C., they survive from ten to twenty-two days. At temperatures in water above freezing, the rate of development increases with the temperature. Apparently, 37 C. is above the optimum temperature for survival of eggs, although when embryonated, they resist that temperature successfully.

Preinfective larvae are more rapidly killed by freezing and by high temperature than are eggs. At 5 C. in shallow water, they may survive between one and two months. Of the temperatures tested, 27 C. is optimal for their development.

Infective larvae are highly resistant to temperature extremes, but if frozen in ice at –6 C., they are killed within approximately two weeks. At a temperature just above freezing, they may survive over 271 days exposure when in water 5 mm. deep. Above 0 C., the mortality increases with rising temperatures.

The following are listed in the order of increasing resistance to desiccation: (1) preinfective larvae, (2) nonembryonated and embryonated eggs, (3) infective larvae. Infective larvae are far more resistant to drying than any of the preinfective stages. They can survive forty-three days exposure to a saturation deficiency of 19.8 mm. of mercury at a temperature of 27 C.

Vertical migration of infective larvae above the soil surface is apparently due to a negative geotropism which in turn is influenced by such factors as light intensity, moisture, and temperature.

Eggs in feces buried to a depth of 12 in. in soil are able to complete their development, and resulting infective larvae can migrate to the surface soil within a few days. The number of larvae developing and reaching the soil surface is inversely proportional to increasing heaviness of the soil. The infective larvae are able to migrate only through soil which is appreciably moist.

Plowing of infested pasture must be considered an inadequate method of ridding it of *O. circumcincta*.

Infective larvae migrate downward in loose soil to a slight extent. This may act as a protective mechanism during unfavorable weather.

Lateral migration of larvae on surface soil is limited to a radius of 1 to 2 in.

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A Bacteriological Study of the Infections Which Follow Injury to the Bovine Udder

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THE INJECTION of mastitis organisms into the teat canal or milk cistern readily produces infection of the bovine udder.* However, external exposure of the intact teat meatus to mastitis bacteria is apparently not in itself sufficient to establish the organisms in the gland. Under experimental conditions imitating natural exposures such as milking with contaminated hands, using infected bedding, or even the application of cultures to the teat opening, transmission rarely occurs.† The closing mechanism of the teat appears to be an effective barrier to the entrance of bacteria into the udder.

Only when the teat is injured does external exposure readily induce infection of the gland. Experiments have been described in which the teat was exposed to infection after the meatus had been injured by laceration with a scalpel, by dilation with surgical instruments sufficient to draw blood, or by bruising with blunt instruments. After such injury the external exposure of the teat to mastitis organisms usually resulted in infection of the quarter. These experiments suggest that injury to the teat may be necessary to cause infection of the gland.

The importance of udder injuries has been recognized by many writers.‡ Dairymen are advised to provide roomy stalls and partitions between stalls in order that there will be less likelihood of cows stepping on teats. It is pointed out that deep gutters and high door sills may be the source of udder injuries. Owners are warned against driving cows too fast, using insufficient bedding, leaving milking machines on after the udder is emptied, and milking with high vacuum. The use of milking tubes and teat dilators is discouraged.

Many conditions other than injury have been suggested as playing a rôle in establishing infections of the bovine udder. Among the conditions suggested have been the following: suction during milking,[4,6,16] incomplete milking,[4,26] composition of the feed,[23] exposure of the udder to cold,[4,12,26] the anatomy of the udder,[1,4,16] heredity,[28,29] sensitization of the udder by repeated exposure to infection,[18] the bactericidal property of milk,[17] and nonspecific mastitis.[24]

Because of the apparent importance of udder trauma in establishing mastitis bacteria in the udder, this study of udder injuries occurring under field conditions was undertaken. The incidence of infection in 317 cases of udder injury is reported. The types of organisms found will be compared with those of acute, subacute, and chronic mastitis.

METHODS

Samples of the secretions from 317 quarters showing evidences of injury were taken for bacteriological examination. These samples, from 119 herds, were obtained by S. D. Johnson, of the Department of Medicine, New York State Veterinary College. Most of the examinations were made within two weeks after the time of injury. Milk was drawn from injured quarters irrespective of other conditions such as clinical evidence of disease and may be considered as unselected. Repeated samples were taken in some cases. Frequently, milk was obtained from all of the quarters of the cows with udder injuries.

The gross appearance of the sample was noted; the reaction to bromthymol blue was recorded; the presence of chlorides greater or less than 0.14 per cent was determined; cell counts were made; the samples were plated in a 1:10 and frequently in higher dilutions in beef-infusion agar containing 8 per cent de-

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*Ref. 5, 7, 13, 21.

†Ref. 1, 2, 5, 7, 20, 21, 25.

‡Ref. 1, 3, 4, 8, 11, 12, 22, 26, 27.

fibrinated horse blood, and smears of the samples incubated at 37 C. for twenty-four hours were examined microscopically.

The methods used for the identification of the bacteria have been described.[9]

INCIDENCE OF INFECTION FOLLOWING INJURY

The observation is commonly made by dairymen that mastitis is likely to follow injury to the cow's teat. How often infection follows trauma to the udder is a matter of particular interest in this study. Milk from a total of 317 quarters showing evidence of trauma was obtained and examined according to the methods described. These 317 cases involved 284 cows; in a few cows, more than one quarter had been injured.

Of the 317 cases of injured quarters, 283 (89%) were found to be infected following injury. The incidence of infection following injury may be compared with the incidence of infection in the apparently uninjured quarters of these same cows. Of 570 samples from apparently uninjured quarters of these cows, 91 (16%) were infected.

The 89 per cent infection following injury may be compared further with the general incidence of udder infections in dairy herds. Of 3,560 quarters of 892 cows examined in 22 dairy herds, 499 (14%) were infected.

The results of bacteriological examinations made prior to injury likewise suggest that the infection found after injury was associated with the injury. Bacteriological examinations made on 93 of the 283 infected quarters prior to injury showed 84 to be free from infection. Of the 9 quarters which were infected 5 harbored the same organism as was found after trauma and 4 were infected with bacteria which differed from those present after injury.

INJURED QUARTERS REMAINING FREE FROM INFECTION

Most of the cases of udder injuries were followed by infection of the gland. The few cases which remained free from infection are of interest because even though free from mastitis bacteria they may have shown evidence of disturbance such as increased cell count, visibly abnormal milk, or even swelling of the quarter.

Of the 317 cases of udder injuries examined, 34 (11%) did not show infection. Samples were taken from 24 of these cases only once and from 10 of them, samples were taken twice or oftener. A total of 54 samples were examined from these 34 cases which did not become infected after injury.

The evidences of disturbance in the 34 injured quarters which remained free from infection are of interest. Such cases of mastitis may constitute some of the cases in which no bacteria of pathogenic significance can be found. Four of these 34 injured quarters were reported to be swollen. About one half of the milk from the 34 cases was grossly abnormal and contained more than a million cells per cc. The results of the examinations of the samples from these 34 injured quarters which remained free from infection follow:

Gross appearance of the secretions:	
normal	17
bloody	10
off color	2
clots	3
pus present	2
purulent	0
Chloride content:	
less than 0.14%	12
greater than 0.14%	22
Bromthymol blue reaction:	
negative	12
slight light green	7
light green	6
green	9
dark green	0
yellow	0
Cells per cc.:	
<20,000	7
20,000 to 500,000	9
500,000 to 1 million	2
1 million to 5 million	5
5 million to 20 million	5
>20 million	6

The nature of the injuries may offer some explanation for these quarters remaining free from infection. From the descriptions recorded (in 12 cases there were no descriptions), it appears that most of the injuries to these quarters did not involve the teat meatus. In only 3 cases was it likely that the teat opening was involved; in 1 of these cases, the teat was

stepped on; in another case, there were cow-pox lesions at the end of the teat, and in the third case the lesion was merely described as a teat injury. The descriptions of the remaining cases indicate that the teat opening was not affected. Two were described as "cuts on the side of the teat;" 4 as "injuries to the side of the teat;" in 3 cases, the quarters were described as injured so that the milk became bloody; in 2 cases, the quarter had been bumped; in 6, it had been hooked, and in the remaining 2 cases, the quarter was described as "bruised" and "injured high up."

Most injuries were caused by cows stepping on teats. Other injuries were described as cuts, often from barbed wire. Frequently, the stenosis resulting from injury induced the use of milking tubes or dilators, a practice which in itself is almost certain to result in infection of the gland. Some of the injuries here reported were the results of surgical operation on the meatus. Many of the quarters had cow-pox lesions at the end of the teats.

Because no obvious lesions were found from the use of milking machines no cases of injury from this source have been included in this study. However, the swelling of quarters and other evidence of disturbances often found traceable to the milking machine make it likely that the milking machine is a common source of udder injuries. Quarters injured in this manner may be free from bacterial infection, especially when first observed.

TABLE 1—Organisms Present in Various Types of Udder Infections

Type of Infection	No. of Cases	*Str. agalactiae*	*Str. dysgalactiae*	*Str. uberis*	Other Streptococci	Staphylococci	*Co. pyogenes*	Coliform	Mixed
Injury	283	67(24%)	65(23%)	43(15%)	27(10%)	36(15%)	17(6%)	7(2%)	21(7%)
Unselected .	499	285(57%)	17(3%)	42(8%)	8(2%)	142(29%)	2(<1%)	2(<1%)	2(<1%)
Chronic* ..	480	280(58%)	16(3%)	37(8%)	8(2%)	139(29%)	0	0	0
Subacute† .	95	13(14%)	9(9%)	7(7%)	12(13%)	41(43%)	11(12%)	2(2%)	0
Acute†	53	0	5(9%)	4(8%)	9(17%)	8(15%)	4(8%)	22(42%)	1(2%)

*Part of the data on streptococcic infections was taken from reference 9.
†Data from a previous report, reference 10.

Udder Injury in Which Infection Followed

Of the 317 injured quarters examined, 283 (89%) were infected. From the nature of the samples taken from these quarters, it is evident that in most cases there was a pronounced reaction of the gland. The results of the examination of the milk from these 283 cases of infection after injury may be summarized as follows:

Gross appearance of the secretions:	
normal	108
bloody	12
off color	26
clots	40
pus present	30
purulent	67
Chloride content:	
less than 0.14%	17
greater than 0.14%	266
Bromthymol blue reactions:	
negative	40
slight light green	39
light green	59
green	86
dark green	58
yellow	1
Cells per cc.:	
<20,000	2
20,000 to 500,000	10
500,000 to 1 million	22
1 million to 5 million	52
5 million to 20 million	39
>20 million	158

The Organisms Present After Injury

The bacteria isolated from the 283 quarters which were found to be infected after injury are listed in the table. In this same table are shown the organisms present in chronic, subacute, and acute mastitis and the organisms present in unselected cases of udder infections. All of the bacteria occurring in the different types of mastitis cases were found in the injured quarters. It is probable, therefore, that any of the mastitis organisms may be established in the udder through trauma.

The data listed in the table show that infections of the udder differ to some extent according to whether the cases are acute, subacute or chronic. Of the 480 cases of chronic mastitis examined, 58 per cent were infected with *Streptococcus agalactiae*, 29 per cent with staphylococci, 3

per cent with *Streptococcus dysgalactiae*, 8 per cent with *Streptococcus uberis*, and 2 per cent with streptococci of various other types. No *Corynebacterium pyogenes*, coliform, or mixed infections were found in these chronic cases.

Cases of mastitis which may be regarded as subacute appear to be either potentially acute infections or flare-ups of chronic infections. In the 95 cases of subacute mastitis examined, there were 14 per cent *Str. agalactiae*, 9 per cent *Str. dysgalactiae*, 7 per cent *Str. uberis*, 13 per cent other streptococci, 43 per cent staphylococci, 12 per cent *Co. pyogenes*, and 2 per cent coliform infections.

In 53 cases of acute mastitis, *Str. agalactiae* was not found except in one case of a mixed infection in which a beta hemolytic streptococcus was also present. Coliform bacteria and streptococci of diverse types were frequently present in these cases of acute mastitis.

The mastitis organisms of unselected infections of the bovine udder are of interest. In single samples of 892 cows in 22 herds, 315 were found to be infected in 499 quarters. The bacteria present in these 499 infected quarters are listed in the table. These data probably indicate fairly well the incidence of the various organisms in infections of the udder. It should be pointed out, however, that such findings will tend to exaggerate the incidence of the more persistent or chronic infections such as *Str. agalactiae*, and to minimize the incidence of the more transient or acute types of infection such as *Co. pyogenes*.

According to the table, *Str. agalactiae* was present in 285 (57%) of the 499 infections found in 3,560 unselected quarters. These findings are considerably lower than most estimates made of the prevalence of this streptococcus in infections of the udder; most writers consider that *Str. agalactiae* is present in over 90 per cent of mastitis cases. In these 499 unselected infections, staphylococci were next in frequency; these organisms were present in 29 per cent of the infected quarters. The remaining mastitis bacteria occurred only occasionally. Three per cent of the infections were *Str. dysgalactiae;* 8 per cent were *Str. uberis;* 2 per cent were other streptococci, and less than 1 per cent of the infections were *Co. pyogenes*, coliform bacteria, or mixed types.

Str. agalactiae infections were present in 10 of the 22 dairy herds examined. The *Str. uberis* and *Str. dysgalactiae* infections occurred sporadically through most of the herds.

Of the 283 cases of infection present as a sequel to udder injury, *Str. agalactiae* was present in 24 per cent. *Str. dysgalactiae* (23%), and *Str. uberis* (15%) infections were relatively frequent. Other streptococci of diverse types were present in 10 per cent of the cases. Staphylococci were present in 15 per cent, *Co. pyogenes* in 6 per cent, coliform organisms in 2 per cent, and mixed infections in 7 per cent of the injury cases.

INCIDENCE OF STR. AGALACTIAE INFECTION IN THE HERDS

The cases of udder injuries were obtained from 119 herds; sufficient bacteriological examinations were made from other cows in 39 of these herds to show that 16 herds were free from *Str. agalactiae* and that 23 herds were infected with this organism. Two hundred and thirteen of the 283 injury cases were from the 39 herds. Fifty-eight of the 213 cases were due to *Str. agalactiae* and came from herds where *Str. agalactiae* was present. *Str. agalactiae* was not found after injuries in herds known to be free from this infection.

In no case was a *Str. agalactiae* infection established by injury in a herd known to be free from this infection, but injuries occurring in infected herds did not necessarily result in infections with *Str. agalactiae*.

The habitat of *Str. agalactiae* is a problem of importance in the transmission of mastitis and in planning its control. The observation that it is established by udder injury only in herds in which cows infected with this organism are present suggests that the only reservoir for this infection is the udder. This organism probably does not persist for long periods outside of the

udder. *Str. agalactiae* has been isolated from teat cups[15] and from the hands of milkers.[14] This streptococcus has also been cultured from the mouths of calves up to four hours and frequently as long as eight hours after the calves had suckled infected cows.[19]

SUMMARY

Experiments cited indicate that the intact meatus is an effective barrier to the entrance of bacteria.

When the meatus is injured, its external exposure to mastitis organisms readily favors the infection.

The possible importance of udder injuries in establishing the infections of bovine mastitis is considered.

The results of the bacteriological examinations of milk from 317 injured quarters are reported.

Most of the injuries resulted from cows treading on teats. Other causes of trauma were wire cuts, cow pox, surgical operations, and the use of milking tubes and dilators.

Only 34 (11%) of the 317 injured quarters were not infected. Most of the injuries of these 34 quarters which were free from mastitis bacteria did not appear to involve the teat meatus. Although bacteria were absent in these quarters, about one half of the samples were abnormal in appearance, gave a pronounced reaction to the bromthymol blue test, and contained large numbers of cells.

Swelling of the quarter was observed in 4 cases. It is apparent that udder trauma in itself may produce disturbances in the gland resembling those resulting from udder infections.

The observation is made that the occurrence of cases of mastitis without bacterial infection in a herd may be caused by the milking machine.

Two hundred and eighty-three (89%) of the 317 injured quarters were infected. This is compared with 14 per cent infection of 3,560 unselected quarters.

Streptococcus agalactiae, the common organism of bovine mastitis, was present in only 24 per cent of the 283 quarters found to be infected after trauma. *Streptococcus dysgalactiae* was cultured from 23 per cent of the infected injury cases, *Streptococcus uberis* from 15 per cent, and other streptococci of diverse types from 10 per cent. Staphylococci were present in 15 per cent, *Corynebacterium pyogenes* in 6 per cent, coliform organisms in 2 per cent, and mixed infections in 7 per cent.

The bacteria of the 283 infected quarters showing injury are compared with the organisms present in 499 unselected udder infections, 480 chronic infections, 95 subacute infections, and 53 acute infections. It appears that any of the organisms capable of producing disease in the udder may be established by injury to the teat. Most of the milk from these quarters in which infection was found after injury showed evidences of abnormalities in gross appearance, chloride content, bromthymol blue reaction, and cell counts.

Some variation of the type of infection with the nature of the disease was noted. The occurrence of *Str. agalactiae* is of particular interest. This organism was not found to be the cause of acute mastitis, but was present in 14 per cent of the subacute cases and 58 per cent of the chronic ones. Fifty-seven per cent of the infections disclosed in single examinations of 892 cows of 22 herds were *Str. agalactiae*.

The presence or absence of *Str. agalactiae* infections was determined in 39 of the 119 herds from which the injury cases were obtained. *Str. agalactiae* was not found after injuries in herds known to be free from this infection. This observation supports the idea that the udder is the only reservoir of infection for the common organism of bovine mastitis.

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The Hydrogen Ion Concentration of the Preputial Cavity of the Bull

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VERY LITTLE information is available in the literature on the normal hydrogen ion concentration of the preputial cavity of the bull. This information may be of value in the further understanding of the establishment of *Trichomonas foetus* in the bull and is a basic contribution to the physiology of reproduction, therefore, appearing to be worthy of recording. To a certain extent, this work is a continuation of earlier studies by Morgan[1] and Morgan and Whitehair[2, 3] and Whitehair.[4]

METHODS AND MATERIALS

The 30 bulls used in this study were all normal animals as far as could be determined by general appearance, breeding history, and microscopic examination of their semen. The bulls were secured in a stall and properly restrained by an assistant using a sideline to reduce any exertion to a minimum.

The pH determinations were made with a Coleman 200 electrometer rewired to accommodate a Coleman 3302 extension glass electrode with a 6-ft. wire lead. The glass electrode, which is 1/4 in. by 3/8 in., is encased in a bakelite tube, 9 in. long and 5/16 in. in diameter. A strong rubber tube about 18 in. long was attached to facilitate placing the glass electrode in the desired positions.

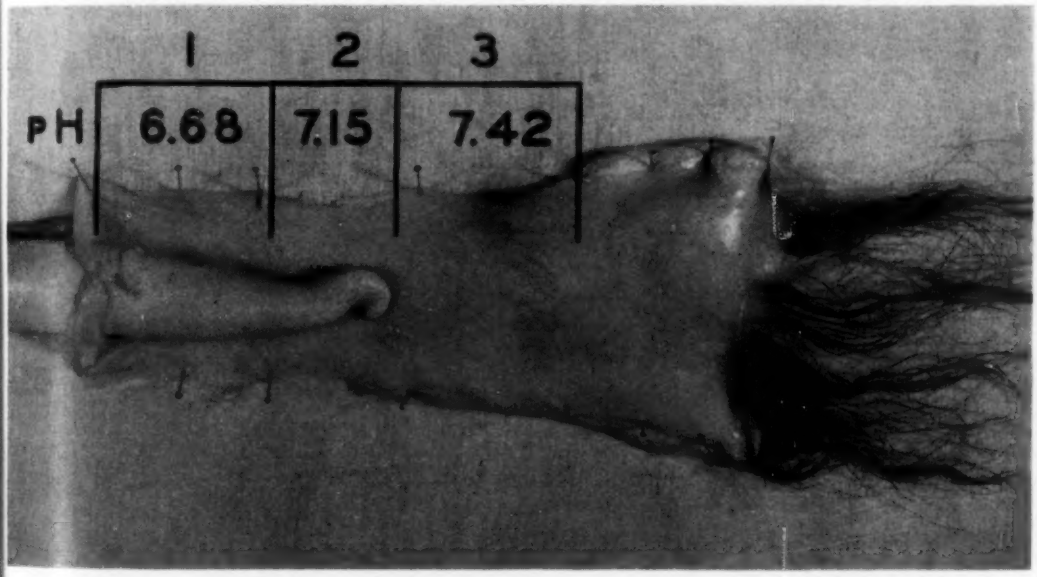

Fig. 1—The preputial sac photographed to show the 3 areas in which the pH readings were made: (1) posterior area of the glans penis not including the galea; (2) area of the galea glandis or anterior portion of the penis; (3) the middle area of the prepuce. The mean pH of each area is also shown.

The determinations were made in three areas of the preputial cavity, namely, (1) posterior part or the portion of the glans penis not including the galea, (2) the galea glandis or anterior portion of the penis, and (3) the

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From the Department of Veterinary Science, University of Wisconsin.

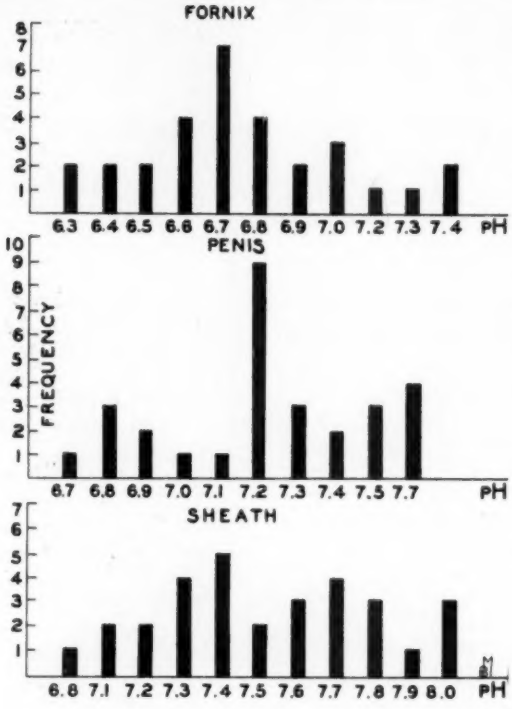

Fig. 2—Frequency distribution of pH of 3 areas of the preputial cavity compiled from 30 bulls.

middle area of the prepuce (fig. 1). The first two areas were chosen because it has been shown by Hammond and Bartlett,[5] Morgan and Whitehair,[6] Abelein,[7] and Andrews and Miller[8] to be the preferential site of *T. foetus* in the bull.

The preputial orifice was washed and dried. The electrode was inserted in the previously mentioned areas and two to three readings were recorded within two minutes. The last reading was used to compute the means. Immediately after withdrawal, the electrode was checked against a standard buffer for accuracy of the apparatus. The electrode was thoroughly cleaned and rinsed in neutral distilled water between animals.

The hydrogen ion concentration is expressed as pH; numbers less than 7.0 are acid, 7.0 neutral, and numbers more than 7.0 are alkaline. In the computation of the means, the pH values were converted into hydrogen ion concentration expressed as Gm. ions per liter. The means were then reconverted into pH values.

RESULTS

In the preputial area posterior to the galea glandis, the lowest pH reading was 6.3 and the highest 7.4. The total mean pH for this area was 6.68. In the region of the galea glandis or the anterior end

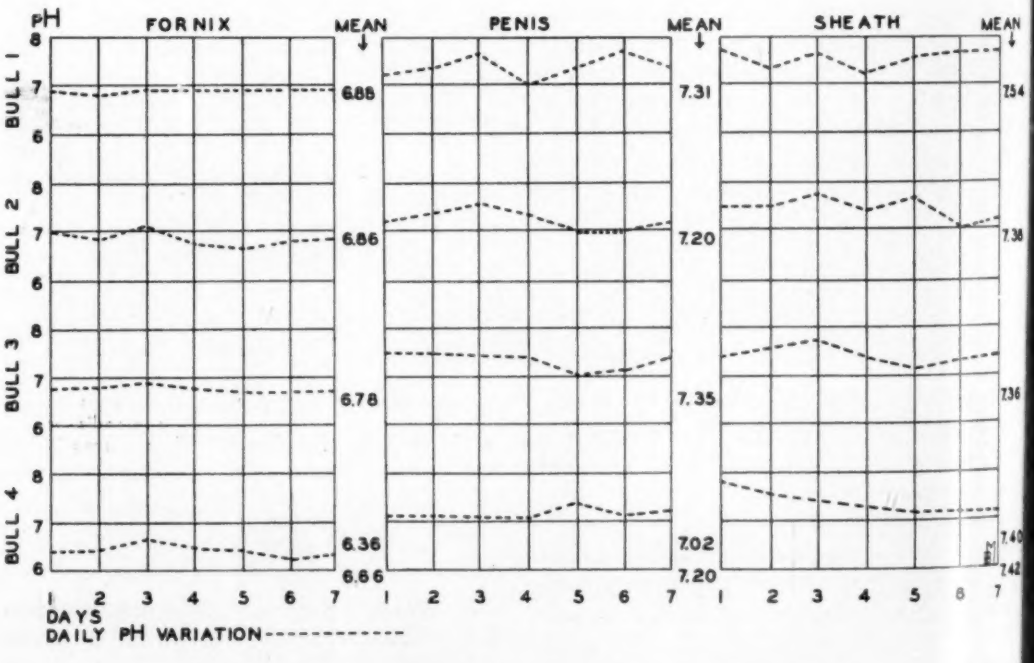

Fig. 3—Daily pH variations of the preputial cavity of 4 bulls for 7 days.

of the penis, the lowest pH reading was 6.7 and the highest 7.7, with a total mean of this region at 7.15. In the middle portion of the prepuce, the lowest pH reading was 6.8 and the highest 8.0. The total mean pH for the sheath was 7.42. It will be noted in figure 2 from the frequency distribution chart, that the pH values lie between 6.6 and 6.8 for the preputial area posterior to the galea glandis; around 7.2 for the anterior end of the penis; between 7.3 and 7.4 for the middle portion of the prepuce.

Figure 3 shows the daily pH variations of the preputial cavity of 4 bulls for seven days. There appears to be no significant difference in the daily variation of the pH. There was more fluctuation in the daily pH value in the middle of the prepuce than elsewhere. This may have been due to urine, semen, or an accumulation of extraneous débris.

SUMMARY

The hydrogen ion concentration was determined for three different regions of the preputial cavity of 30 normal bulls. The pH readings of these same areas were studied for seven days to determine the daily variation. The pH of the posterior preputial cavity was found to be between 6.3 and 7.4, with a total mean value of 6.68. In the region of the preputial cavity near the anterior end of the penis, the pH varied from 6.7 to 7.7, with a total mean pH of 7.15. The pH readings in the middle of the prepuce were between 6.8 and 8.0, with a total mean of 7.42. No significant daily variation in the hydrogen ion concentration of the preputial cavity of bulls was observed.

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